A STUDY OF GENETIC VARIABILITY AT THE *CYP11B2/B1* LOCUS AND ITS IMPORTANCE IN HUMAN HYPERTENSION

by

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ABSTRACT

Essential hypertension is a complex multifactorial condition with a known genetic component. Several lines of evidence suggest a contributory role of adrenal cortex function to the heritable component of hypertension.

In the human adrenal cortex, the enzymes 11β-hydroxylase and aldosterone synthase are responsible for the final stages in the synthesis of cortisol and aldosterone respectively. The gene encoding 11β-hydroxylase (*CYP11B1*) lies in tandem with the gene encoding aldosterone synthase (*CYP11B2*) on chromosome 8. The two genes are highly homologous with the main differences being in their regulatory regions, as ACTH primarily regulates *CYP11B1* whereas *CYP11B2* is under the control of angiotensin II and potassium.

Variation within these genes has been implicated in certain forms of hypertension. Mutations in *CYP11B1* and *CYP11B2* account for the biochemical and haemodynamic abnormalities in the Dahl salt-sensitive rat. In humans, mutations in *CYP11B2* and/or *CYP11B1* lead to the rare clinical syndromes of glucocorticoid-suppressible hyperaldosteronism and 11β-hydroxylase deficiency that are associated with mineralocorticoid hypertension. For these reasons, previous studies have examined whether polymorphisms associated with *CYP11B2* are involved in hypertension. Several studies have reported that variation within the 5'UTR (-344 C/T) and intron 2 (intron conversion (IC)) are associated with increased aldosterone levels and increased blood pressure. However, these findings have not always been confirmed and the -344 C/T variation seems to have no influence on aldosterone biosynthesis *in vitro*. Paradoxically, it is more closely associated with markers of an impaired 11β-hydroxylase activity, which draws attention to the homologous gene *CYP11B1*. The studies reported in this thesis aimed to identify the pattern of variation across the *CYP11B1/CYP11B2* locus in order to determine the genetic variation responsible for the

observed impaired 11β-hydroxylation and its link with aldosterone levels and hypertension.

In **Chapter 3,** an attempt was made to identify polymorphisms in *CYP11B1* that associate with hypertension and a raised aldosterone-to-renin ratio (ARR) and therefore might contribute to the genetic component of these phenotypes. No polymorphism was identified that could account for either phenotype. However, eight novel missense mutations were identified out of 160 subjects screened, indicating a high sequence variation. These were identified in exons 2 and 3 of the *CYP11B1* gene and alter the encoded amino acids; R43Q, L83S, H125R, P135S, F139L, L158P, L186V and T196A. These variants were recreated *in vitro* to examine their effects on enzyme function. Enzyme activity was assessed by conversion of 11-deoxycortisol (S) to cortisol (F) and 11-deoxycorticosterone (DOC) to corticosterone (B).

The mutants L158P and L83S almost abolished enzyme function whilst R43Q, F139L and P135S and T196A enzymes resulted in product levels that were approximately 30% to 50% that of wildtype levels. The mutant enzymes H125R and L186V resulted in substrate-specific alterations in enzyme function. H125R decreased conversion of S to F and L186V increased S conversion. Neither had an effect on the conversion of DOC to B. An attempt was made to assign these residues to locations within the protein molecule using homology-modelling techniques. This confirmed that none of the mutations are located within the putative active site. The importance of these affected residues, remote from the active site, to enzyme function, will inform subsequent studies of structure/function relationships.

The study in **Chapter 4** aimed to analyze the pattern of variation across the *CYP11B2/B1* locus in detail. This confirmed that the overall sequence variation across the *CYP11B* locus is high and that there is tight linkage disequilibrium across the entire

region with only 4 common haplotypes accounting for 68% of chromosomes in the data set. The detailed sequencing identified several candidate polymorphisms that could account for the observed phenotypes, including increased aldosterone levels and inefficient 11β-hydroxylase function, previously associated with the -344 C/T polymorphisms in *CYP11B2*. The polymorphisms identified within the coding regions of the two genes were either synonomous (*CYP11B2* Ser169Ser and Arg374Arg, *CYP11B1* Leu75Leu and Asp82Asp) or are known to have no affect on activity (*CYP11B2* Lys173Arg). The majority of variants were identified in the introns and 3'UTR of *CYP11B2* and *CYP11B1* and their functional effects remain to be determined. Of particular interest, due to their potential to alter gene transcription, was the identification of 2 in the 5' regulatory region of *CYP11B1* (-1889 G/T and -1859 A/G) and 7 within the 5' regulatory region of *CYP11B2*.

In **Chapter 5**, the frequency and phenotypic associations of the newly identified *CYP11B1* 5'UTR polymorphisms -1889 G/T and -1859 A/G were investigated in a hypertensive population. The frequencies were found to be -1889 G-0.53, T- 0.47; -1859 A-0.5, G-0.5. Interestingly, the -1889 G/T and -1859 A/G polymorphisms were found to associate with an impaired 11β-hydroxylase efficiency in a hypertensive population. The THS/total F ratio (index of 11ß-hydroxylase activity) was significantly higher in -1889 TT homozygotes than in GG homozygotes ($p = 0.025$). A similar pattern was seen with the -1859 SNP, with GG homozygotes tending to have a higher ratio than AA homozygotes ($p = 0.056$).

These steroid data were supported by the results from **Chapter 6** that examined the effects of these polymorphisms *in vitro* using luciferase reporter gene constructs transfected into Y1 mouse adrenal cells. There was no significant effect on basal expression levels. However, the construct containing the -1889 T and the -1859 G alleles had a significantly reduced response to stimulation with either 1μM ACTH ($p <$

0.001) or 10μM Forskolin ($p = 0.036$) when compared to the contrasting allele combination.

Evidence of a slightly reduced 11β-hydroxylase efficiency is a consistent finding in subjects with essential hypertension when compared to normotensive controls. However, preliminary investigation of the -1889 G/T and -1859 A/G polymorphisms in a hypertensive case-control population failed to show any association with hypertension (**Chapter 5**).

In summary, these studies have confirmed that the *CYP11B2* and *CYP11B1* genes contain a high frequency of genetic variation. Many of the variants identified in *CYP11B1* have been shown to alter activity significantly. There is strong evidence to suggest that the impaired 11β-hydroxylase efficiency previously associated with the -344 C/T and IC variants in *CYP11B2* is due to linkage with the -1889 G/T and -1859 A/G polymorphisms upstream of *CYP11B1*. However, the causative link with hypertension and a raised ARR remains to be determined.

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PUBLICATIONS

Barr M, MacKenzie SM, Wilkinson DM, Holloway CD, Friel EC, Miller S, MacDonald T, Fraser R, Connell JMC, Davies E (2006) Functional effects of genetic variants in the 11b-hydroxylase (CYP11B1) gene. *Clinical Endocrinology* 65: 816-825.

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DECLARATION

I declare that the work presented in this thesis is, to the best of my knowledge and belief, original and my own work, unless specified otherwise in the text.

Marianne Barr

DEFINITIONS

3βHSD	Gene encoding 3β-hydroxysteroid dehydrogenase
11β-HSD	11β-Hydroxysteroid dehydrogenase
17-OH-P	17-Hydroxyprogesterone
17-OH-PREG	17-Hydroxypregnenolone
18-OH-B	18-Hydroxycorticosterone
AC	Adenylyl (adenylate) cyclase
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotropin
ACTH-R	ACTH receptor
A'dione	Androstenedione
AIP	Aldosterone induced protein
Aldo	Aldosterone
AME	Apparent mineralocorticoid excess
Ang I	Angiotensin I
Ang II	Angiotensin II
AP-1	Activator protein 1
ARR	Aldosterone-renin ratio
AT1 or 2	Angiotensin II receptor 1 or 2
ATF-1	Activating transcription factor 1
ATP	Adenosine triphosphate
B	Corticosterone
BAH	Bilateral adrenal hyperplasia
BMI	Body mass index
BSA	Bovine serum albumin
C	Cholesterol
Ca^{2+}	Calcium ions
$[Ca^{2+}]_i$	Intracellular free calcium concentration
CaMK	Ca^{2+}-calmodulin-dependent protein kinase
lipoid CAH	Congenital adrenal lipoid hyperplasia
cAMP	3', 5'-Cyclic adenosine monophosphate
CBP	CREB binding protein
cDNA	Complementary deoxyribonucleic acid
CHIF	Corticosteroid hormone induced factor
Cl^-	Chloride ions
CMOI/II	Corticosterone methyl oxidase type I or II

CNS	Central nervous system
COUP-TF	Chicken ovalbumin upstream promoter-transcription factor
CRE	cAMP-response element
CREB	cAMP-responsive element binding protein
CRH	Corticotrophin-releasing hormone
CYP11A1	Gene encoding side-chain cleavage enzyme
CYP11B1	Gene encoding 11β-hydroxylase
CYP11B2	Gene encoding aldosterone synthase
CYP17	Gene encoding 17-hydroxylase
CYP21	Gene encoding 21-Hydroxylase
CYP450	Cytochrome P450 enzymes
DAG	1,2-Diacylglycerol
DBD	DNA binding domain
DBI	Diazepam-binding inhibitor
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
DOC	11-Deoxycorticosterone
DOCA	Deoxycorticosterone acetate
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminotetraacetic acid
EMSA	Electrophoretic mobility shift assay
E	Cortisone
ENaC	Epithelial sodium channel
EtOH	Ethanol
F	Cortisol
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
G protein	Guanine nucleotide-binding regulatory protein
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GSH	Glucocorticoid suppressible hyperaldosteronism
GTP	Guanosine triphosphate
HDL	High-density lipoproteins
HPA	Hypothalamic-pituitary-adrenal axis

HPLC	High performance liquid chromatography
HRE	Hormone response element
icv	Intracerebroventricular
IP_3	1, 4, 5, Inositol triphosphate
K^+	Potassium ions
LB	Luria-Bertani
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
LV	Left ventricle
LVEDP	Left ventricular end-diastolic pressure
LVH	Left ventricular hypertrophy
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
Na^+	Sodium ions
NAC	N-acetylcysteine
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
Na^+/K^+-ATPase	Sodium, potassium-adenosine triphosphatase
nfH_2O	Nuclease free water
NGFIB	Nerve growth factor-induced clone B
NMDA	*N*-Methyl-D-asparate
NO	Nitric oxide
NOS	Nitric oxide synthase
NURR1	NUR-related factor 1
P	Progesterone
$P450_{11\beta}$	11β-Hydroxylase
P450c17	17-Hydroxylase
P450c21	21-Hydroxylase
$P450_{aldo}$	Aldosterone synthase
P450scc	Side-chain cleavage enzyme
PBR	Peripheral-type benzodiazepine receptor
PBS	Phosphate-buffered saline
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C

POMC	Pro-opiomelanocortin
PREG	Pregnenolone
Ptx1	Pituitary homeobox 1
QTL	Quantitative trait loci
RAS	Renin-angiotensin system
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription-polymerase chain reaction
S	11-Deoxycortisol
SAP	Steroidogenesis activator peptide
SCP-2	Sterol carrier protein-2
SF-1	Steroidogenic factor 1
Sgk1	Serum and glucocorticoid-regulated kinase 1
SIP	Steroidogenesis-inducing protein
SNP	Single nucleotide polymorphism
StAR	Steroidogenic acute regulatory protein
TAE	Tris-acetic EDTA buffer
TBS	Tris-buffered saline
TE	Tris-HCl EDTA buffer
THE	Tetrahydrocortisone
THF	Tetrahydrocortisol
THS	Tetrahydrodeoxycortisol
VSMC	Vascular smooth muscle cell

CHAPTER 1

The Role of Corticosteroids in Essential Hypertension

1.1 Essential Hypertension

Essential hypertension or hypertension of unknown cause accounts for more than 90% of cases of hypertension (Oparil *et al* 2003). It is a major clinical problem affecting over 25% of the adult population worldwide (Kearney *et al* 2005). For diagnostic purposes hypertension is defined as resting blood pressure levels repeatedly above 140/90mm Hg in adults. This is more common in men than in women and also in people aged over 65 than in younger people. Blood pressure is a quantitative trait showing a continuous distribution within the population. Therefore there is no logical sharp boundary between 'normal' and 'abnormal' values. Essential hypertension can be defined as the blood pressure value associated with an inceased risk of cardiovascular disease. At this elevated level of blood pressure, the risk of myocardial infarction, stroke and heart failure is greatly increased (Mosterd *et al* 1999) and the higher the blood pressure, the worse the disease. Cardiovascular diseases are responsible for approximately a third of all deaths (Kearney *et al* 2005) and so adequate control of blood pressure within the population is a very important goal both clinically and economically. The current understanding of essential hypertension is that it is a complex condition resulting from the interaction of multiple genetic and environmental factors. However, the identity of the genes that predispose to hypertension remains largely unknown. A better understanding of the contributing genetic factors would provide sites for targeted pharmacotherapy.

In the following sections of this review, I wish to explore the evidence for a contributory role of adrenal cortex function to the heritable component of hypertension. I shall discuss briefly the general factors predisposing to hypertension including the evidence for a general genetic component. The structure of the adrenal

gland and the biosynthestic pathways for the synthesis of its principal corticosteroid hormones, cortisol and aldosterone will be described. The major effects of these hormones and their mechanisms of action will be explained with particular attention to the ways in which they influence blood pressure. A description of clinical syndromes of corticosteroid deficiency or excess highlights their importance in blood pressure regulation. However, to address the main aim of this review which is to evaluate the contribution of adrenocortical function to the heritable component of hypertension requires a detailed look at the molecular genetics of aldosterone and cortisol synthesis. In particular, focusing on gene and enzyme structure and regulation of expression. The strong evidence from both animal models and rare monogenic forms of human hypertension, that modification of these genes affect blood pressure and cardiovascular function is presented. Finally there is evidence to suggest that similar genetic variation is associated with the increasingly common syndrome of essential hypertension.

From this review I will present the hypothesis that genetic variation within the genes responsible for aldosterone and cortisol synthesis act not only independently to increase risk but also interact in a previously unsuspected way. This will form the basis of my experimental work.

1.2 Environmental Risk Factors

Evidence suggests that hypertension results from an interaction between genetic and environmental risk factors and that this accounts for the high prevalence of essential hypertension in industrialized countries (Kornitzer *et al* 1999). The main modifiable risk factors are: high salt intake, alcohol consumption, obesity and low physical activity. It seems likely that changes in these risk factors have made an important

contribution to the rapid rise in the frequency of hypertension over recent years. The evidence for the contribution of each of the main risk factors is reviewed briefly below.

1.2.1 Salt Intake

The link between salt intake and blood pressure seems predictable from the relationship between salt and vascular volume homeostasis. In 1972, Dahl found a correlation between urinary salt excretion and prevalence of hypertension in humans (Dahl 1972). This association was confirmed by the large international epidemiological study, INTERSALT, which revealed an association, in 52 populations, between sodium excretion and the increase in blood pressure with age. Sodium excretion and systolic blood pressure were also found to be related after adjustment for age, gender, body mass index (BMI), alcohol and potassium intake (Stamler *et al* 1989). Several meta-analyses of randomized clinical trials have confirmed that decreasing salt consumption reduces blood pressure. One meta-analysis comparing randomised clinical trials of hypertensive subjects with those of normotensive subjects concluded that salt reduction resulted in a significant decrease in blood pressure in hypertensive individuals and a small non-significant fall in normotensives (Kornitzer *et al* 1999). However, dietary sodium intake does not raise blood pressure in everyone, and distinction has been made between 'salt sensitive' and 'salt resistant' individuals. This phenotype is also observed in animal models of hypertension such as the Dahl salt sensitive hypertensive rat (Cicila *et al* 1993) (see **section 1.15.1.1**).

1.2.2 Potassium Intake

The INTERSALT study also showed that potassium has a significant inverse relationship with blood pressure in individuals after correction for confounding factors. Therefore, the sodium/potassium ratio is significantly positively related to blood pressure (Stamler *et al* 1989). This inverse relationship of potassium to blood pressure was confirmed in the Multiple Risk Factor Intervention trial (Stamler *et al* 1997). In an interventional study it was demonstrated that a diet of foods with high potassium content such as fruit and vegetables significantly reduced blood pressure compared with a normal diet (Appel *et al* 1997).

1.2.3 Alcohol Consumption

The affect of alcohol intake on blood pressure depends on age and gender (Kornitzer *et al* 1999). Long-term high alcohol consumption increases blood pressure but the precise nature of the relationship varies between studies. The INTERSALT study found an association between high alcohol intake ($\geq$ 300 ml/week) and the prevalence of hypertension after adjusting for age, sex, BMI and urinary Na^+ and K^+ excretion (Stamler *et al* 1989). In a study of male subjects, alcohol intake could explain between 7 and 11% of the occurrence of hypertension (MacMahon 1987). Reducing alcohol intake has a favourable affect on both systolic and diastolic blood pressure in hypertensive subjects (Puddey *et al* 1987).

1.2.4 Obesity

Many population studies have found a significant association between BMI and blood pressure (Kornitzer *et al* 1999). The INTERSALT study found this to be an independent effect (Stamler *et al* 1989). Other studies have shown a favourable

effect on blood pressure of reduction of calorie intake or body weight in hypertensive subjects (Kornitzer *et al* 1999).

1.2.5 Other Factors

Many other environmental factors can potentially affect a person's blood pressure. For example, a sedentary lifestyle, smoking and stress have all been associated with hypertension although the extent of their contribution is not always clear (Kornitzer *et al* 1999; Odermatt 2004). In particular the contribution of stress to hypertension is unclear as it is hard to define or quantify stress and there is a high degree of variability between individuals.

1.3 Evidence for a Genetic Component

Heritability is defined as the total phenotypic variance in a trait that is due to additive genetic effects. Although environmental factors are known to play an important role in the pathogenesis of essential hypertension, evidence from family and epidemiological studies suggests that 30-60% of blood pressure variation within a population is genetic (Ward 1990).

1.3.1 Family Studies

Population studies such as the one conducted in Tecumseh, Michigan, demonstrate that there is a greater similarity of blood pressure within families than between families (Longini, Jr. *et al* 1984). This is not simply due to a shared environment as there is a significant correlation of blood pressure between first-degree relatives but not between spouses who have been sharing the same environment for 10 years or more (Havlik *et al* 1979; Miall *et al* 1967). This is confirmed in adoption studies

where there is a significant correlation of blood pressure between parents and natural children or between natural siblings but not between these and adopted children living in the same household (Biron *et al* 1976). The correlation of blood pressure is higher between monozygotic than dizygotic twins, strongly suggesting that a proportion of the population variation of blood pressure is genetically determined (Feinleib *et al* 1977). The genetic contribution can be quantified using the sibling to risk ratio (lambda-S). This compares the prevalence of hypertension in sibships to the prevalence in the general population. A lambda-S > 2 is believed to indicate a significant genetic component. The lambda-S for hypertension is roughly 3.5 (Jones 1999).

1.4 Identifying the Genes Involved

Having established that essential hypertension is a complex quantitative trait with a strong genetic component, there are several methods to determine which genes (or quantitative trait loci (QTL) contribute to blood pressure). The two main methods for identifying the genes involved are genome wide scans and candidate gene studies. For human subjects, investigations are conducted either as family studies consisting of affected members (usually sibling pairs) or as association studies where groups of hypertensive and normotensive subjects are compared.

A genome wide scan uses random genetic markers spread over the entire genome to identify a QTL that associates with blood pressure. This method allows the detection of novel susceptibility genes that a candidate gene approach would miss. However, an identified QTL will typically consist of a large chromosomal region containing 100-300 genes and further, more stringent linkage mapping must be performed to

reduce the locus and focus on fewer relevant genes. Each of these genes must then be examined for variants.

The candidate gene approach is based on a prior knowledge of the pathophysiology of hypertension, which will suggest potential causative genes. There are several lines of evidence suggesting a contributory role of adrenal corticosteroids in essential hypertension and therefore that the genes encoding the enzymes for their biosynthesis may be key candidates.

1.5 Structure of the Adrenal Gland

The adrenal glands are paired pyramidal-shaped organs situated above each kidney. Each comprises an inner medulla and a surrounding cortex (see **figure 1.1**). The cortex is divided into 3 layers: zona glomerulosa, zona fasciculata and the zona reticularis. These 3 zones are histologically and enzymatically distinct with the steroid hormones produced by each of the zones being dependent on which enzymes are present in the cells of that zone.

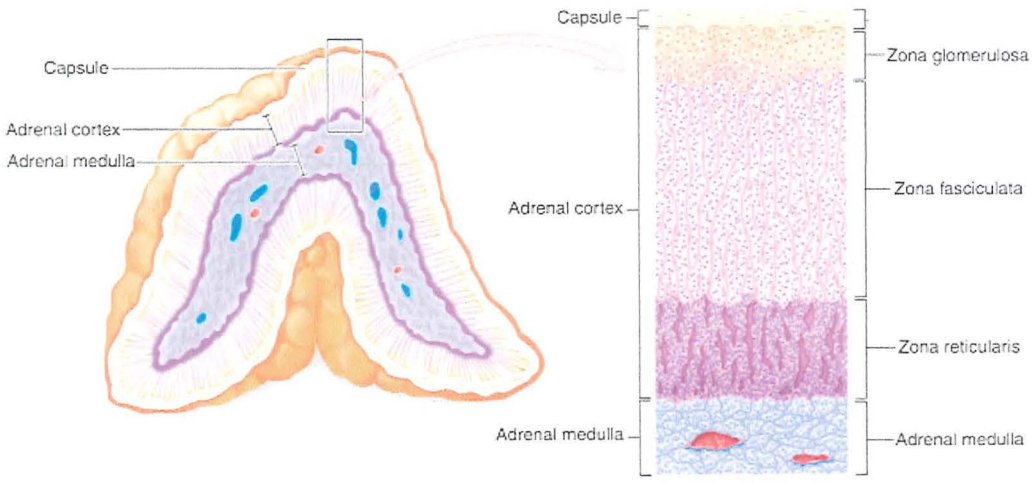


Figure 1.1. Cross section of the adrenal gland showing the distinct layers of the adrenal cortex

(Taken from the McGraw-Hill online learning center; Ch20 Endocrine System http://highered.mcgraw-hill.com)

1.6 Corticosteroids

Corticosteroids are hormones produced by the adrenal cortex that are essential for life. The adrenal corticosteroids secreted by the cortex are divided into two main groups: the mineralocorticoids and the glucocorticoids. The mineralocorticoid aldosterone is synthesised in the zona glomerulosa and affects water and electrolyte homeostasis. The glucocorticoids, cortisol in man and corticosterone in the rat, are synthesised in the zona fasciculata and control carbohydrate, fat and protein metabolism as well as contributing to the control of the immune response. The zona reticularis is the site of sex steroid synthesis (mainly the androgen precursors dehydroepiandrosterone (DHEA) and androstendione (A'dione)).

Corticosteroids are a group of C21 steroids characterized by a double bond at C-4, a C-21 hydroxyl group and an oxo group at C-20. A hydroxyl group or an oxo group may or may not be present at C-11. The presence or absence of a hydroxyl group at C-17 differentiates the 17-oxygenated corticosteroids, such as cortisol or 11-deoxycortisol (S) from the 17-deoxycorticosteroids such as 11-deoxycorticosterone (DOC), corticosterone (B) and aldosterone. The chemical and common names for some of the adrenal steroids are listed in **table 1.1** and the structure of the more important ones are shown in **figure 1.2**. It is possible to relate these structural features to their biological activities. For example the presence of hydroxyl groups at C-17 and C-11 enhances glucocorticoid activity so that cortisol is the most active, whereas absence of a C-17 hydroxyl group results in mineralocorticoid activity eg DOC. Aldosterone contains a hydroxyl group at C-11 but is unique in that it contains an aldehyde group on C-18. The close proximity of these groups results in

hemi-acetyl formation, reducing the influence of the C-11 hydroxyl group and hence decreasing glucocorticoid activity.

All of the adrenal steroids are synthesised from a common substrate, cholesterol, by a series of enzymatic conversions performed by two types of enzyme: cytochrome P450 and hydroxysteroid dehydrogenase enzymes.

Common name	Abbreviation	Systematic name
cholesterol	C	cholest-5-en-3β-ol
pregnenolone	PREG	3β-hydroxypregn-5-ene-20-one
progesterone	P	pregn-4-ene-3,20-dione
17-hydroxypregenolone	17-OH-PREG	3β,17α-dihydroxypregn-5-ene-20-one
17-hydroxyprogesterone	17-OH-P	17α-hydroxypregn-4-ene-3,20-dione
dehydroepiandrosterone	DHEA	3β-hydroxyandrost-5-ene-17-one
androstenedione	A'dione	androst-4-ene-3,17-dione
11-deoxycortisol	S	17α,21-dihydroxypregn-4-ene-3,20-dione
cortisol	F	11β,17α,21-trihydroxypregn-4-ene-3,20-dione
cortisone	E	17α,21-dihydroxypregn-4-ene-3,11,20-trione
11-deoxycorticosterone	DOC	21-hydroxypregn-4-ene-3,20-dione
corticosterone	B	11β,21-dihydroxypregn-4-ene-3,20-dione
18-hydroxycorticosterone	18-OH-B	11β,18,21-trihydroxypregn-4-ene-3,20-dione
aldosterone	Aldo	11β,21-dihydroxypregn-4-ene-3,20-dione-18-al

Table 1.1. Common and systematic names and abbreviations of some important adrenal steroids

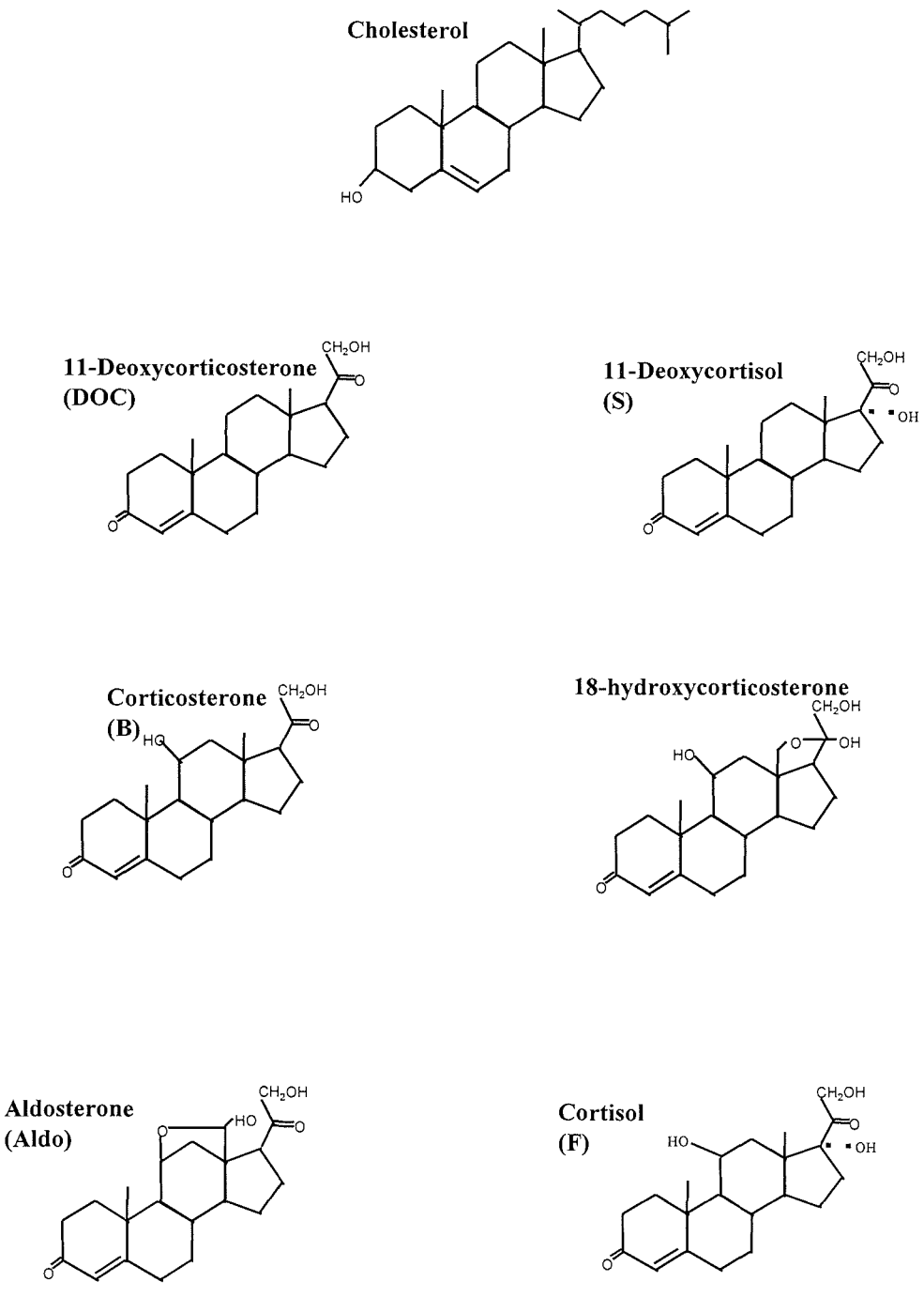


Figure 1.2. Steroid structures and common names

1.7 Cytochrome P450 Enzymes

Cytochrome P450 (CYP450) enzymes form a large superfamily present in all life forms that catalyze the mono-oxygenation (hydroxylation) of a diverse range of substrates. A total of nearly 4000 CYP450 genes have now been identified. Their main functions in humans are xenobiotic metabolism within the liver and steroid synthesis within the endocrine glands such as the gonads and the adrenal cortex. Five CYP450s are involved in the synthesis of steroids in humans.

CYP450 enzymes are mixed function oxygenases that catalyze the incorporation of one oxygen atom from an oxygen molecule into the substrate and reducing the other to water. All these enzymes possess a covalently bound haem prosthetic group to manipulate oxygen and electrons. They are dependant on molecular oxygen (O_2) and electrons donated by NADPH in the following stoichiometry:

$$R\text{-}H + NADPH + H^+ + O_2 \rightarrow R\text{-}OH = NADP^+ + H_2O$$

Each of the steroid hydroxylation reactions involves a two-electron reduction of one molecule of oxygen with concomitant oxygenation of various substrates. The CYP450 enzymes, which are divided into 2 biochemical classes, mitochondrial and microsomal, are the terminal components of an electron transport system. The type I enzymes (side chain cleavage (P450scc), 11β-hydroxylase ($P450_{11\beta}$) and aldosterone synthase ($P450_{aldo}$)) are part of a system located on the inner mitochondrial membrane, comprising NADPH, adrenodoxin reductase (a FAD-containing flavoprotein) and the iron sulphur protein (2Fe-2S) adrenodoxin (see **figure 1.3**). Two high-energy electrons are transferred from NADPH to the FAD of adrenodoxin reductase. Adrenodoxin accepts electrons from adrenodoxin reductase one at a time

and transfers them to the haem iron of the cytochrome P450 enzyme, which is bound to the inner mitochondrial membrane (Bernhardt *et al* 1998). Type II enzymes (21-hydroxylase (P450c21) and 17-hydroxylase (P450c17)) are located within the endoplasmic reticulum and rely on a single NADPH-specific P450 oxidoreductase for the transfer of electrons (see **figure 1.3**). In some cases, this transfer may be assisted by cytochrome b5.

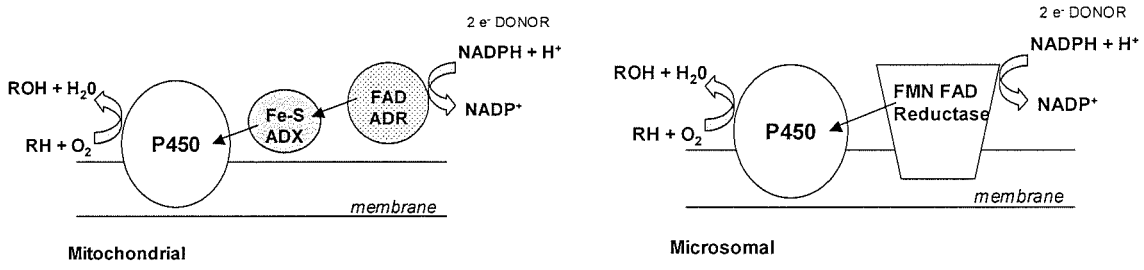


Figure 1.3. Electron transport chains of mitochondrial and microsomal P450 monooxygenases.

Electrons are transferred from NADPH through NADPH-adrenodoxin reductase (ADR) and adrenodoxin (ADX) to mitochondrial P450 or through P450 oxidoreductase to microsomal P450 to exhibit the monooxygenase activity.
RH and ROH indicate the substrate and the product, respectively.
(Adapted from Kominami et al 1984)

The CYP450 reaction involves cyclic oxidation/reduction of the P450-containing haem iron in conjunction with substrate binding and oxygen activation. The cycle is initiated by substrate binding which induces a conformational change that activates the CYP450 from its resting state to form a high-spin ferric (Fe^{3+}) complex on the haem iron. This substrate-bound complex has a much greater +ve reduction potential. The subsequent reduction of the haem iron by two electrons enables oxygen binding, the breaking of the O-O bond within molecular oxygen and the transfer of one oxygen atom to the substrate to yield the oxidised product.

Many CYP450s can bind more than one structurally distinct substrate due to their ability to undergo conformational changes, making them an exception to the classic 'lock and key' concept of enzyme function.

Evidence suggests that adrenodoxin does not form a static ternary complex with adrenodoxin reductase and CYP450 but that it functions as a mobile electron shuttle. Each of these associations is dependent on ionic interactions between complementary charged residues. Site-directed mutagenesis studies have shown that CYP450s interact with adrenodoxin at specific amino acid residues and that changes in the primary sequence of the CYP450 or adrenodoxin can alter the interaction. Factors affecting this interaction, the amount of adrenodoxin or its electron transfer properties can affect the level or pattern of adrenal steroid biosynthesis (Beckert & Bernhardt 1997). The electron transfer proteins are not specific to individual CYP450s. Therefore, adrenodoxin is able to interact with P450scc as well as $P450_{11\beta}$ and $P450_{aldo}$. Competition for electrons from the adrenodoxin, among the P450 enzymes can also alter the pattern of product yields.

1.8 Corticosteroidogenesis

The sequence of reactions which converts cholesterol to corticosteroids in the human adrenal cortex are illustrated in **figure 1.4**. For steroidogenesis to occur, cholesterol must first be delivered to the mitochondrion.

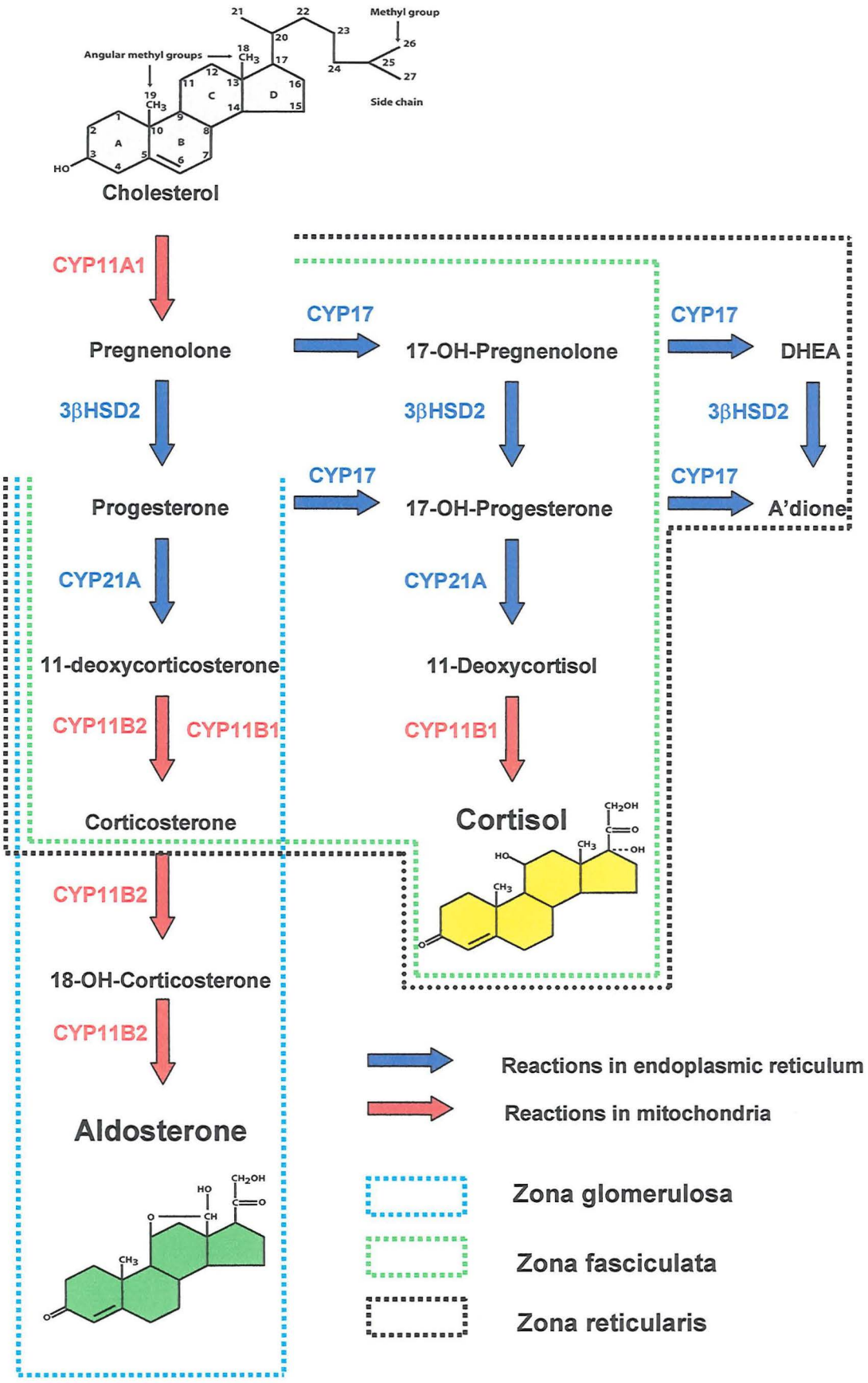


Figure 1.4. Major pathways of corticosteroid biosynthesis in human adrenal cortex.

1.8.1 Cholesterol Uptake

The first stage in the biosynthesis of all adrenal steroids is the cleavage of cholesterols side chain by the cytochrome P450 side chain cleavage enzyme (P450scc), encoded by *CYP11A1*, to produce pregnenolone (see **figure 1.4**). The rate-limiting step of adrenal steroidogenesis is the uptake of cholesterol, which is hydrophobic, across the aqueous intermembrane space of the mitochondrion to the inner mitochondrial membrane where P450scc is located (Black *et al* 1994) (see **figure 1.5**).

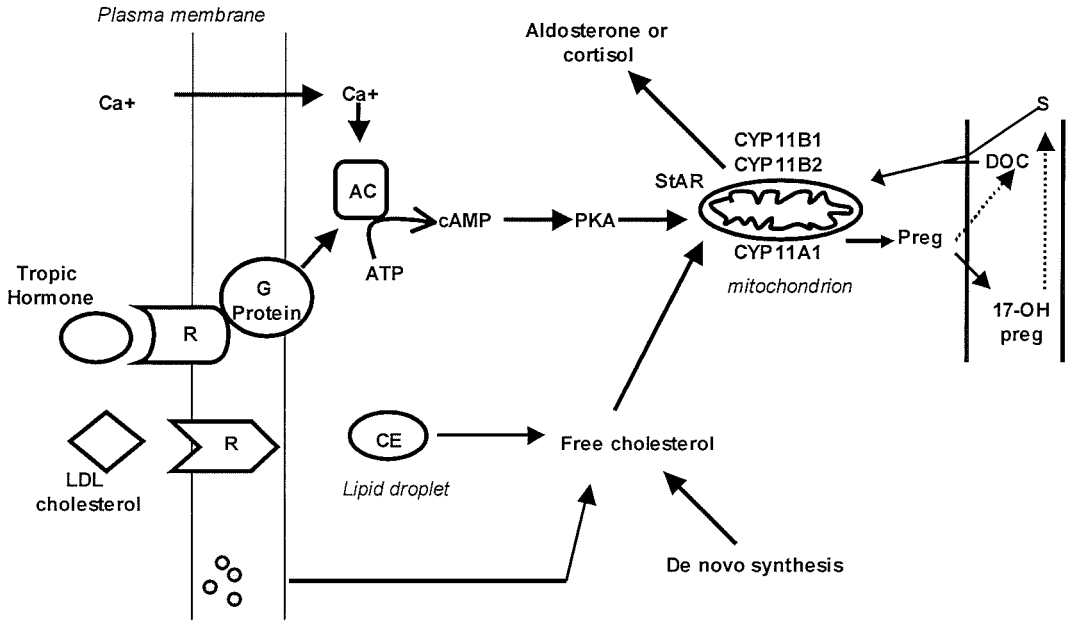


Figure 1.5. Activation of steroid hormone production
(Adapted from Stocco & Clark 1996)

The main supply of cholesterol to adrenal cells in man is from the circulation in the form of low-density lipoprotein cholesterol (Gwynne & Strauss 1982) whereas in rats the main source is from high-density lipoproteins (Gwynne & Mahaffee 1989). The cholesterol can then be stored within the cell in lipid droplet stores as cholesterol

esters. A low level of de novo synthesis of cholesterol from acetate also occurs within the adrenal cortex (Borkowski *et al* 1967). Upon stimulation, these intracellular cholesterol-containing vesicles are transported to the mitochondria along the cytoskeleton fibres of the cells. Free cholesterol is then transported across the intermembrane space to the inner mitochondrial membrane by mechanisms still not well understood. Several putative regulatory proteins have been identified; sterol carrier protein-2 (SCP-2) (Vahouny *et al* 1985), steroidogenesis activator protein (SAP) (Frustaci *et al* 1989; Pedersen & Brownie 1983), the mitochondrial benzodiazepine receptor (PBR) and its endogenous ligand diazepam-binding inhibitor (DBI) (Papadopoulos 1993) and the steroidogenic acute regulatory (StAR) protein which has been the most widely studied and has the most compelling evidence for this role (Clark *et al* 1994). This is considered in more detail below.

1.8.2 Steroidogenic acute regulatory (StAR) protein

StAR is synthesised as a 37-kDa cytosolic precursor with an N-terminal mitochondrial-signalling sequence, which upon hormonal stimulation can be imported and modified by the mitochondria to a mature 30-kDa form (Epstein & Orme-Johnson 1991). Several studies have shown that the synthesis of the 30-kDa protein correlates with the synthesis of steroids (Epstein & Orme-Johnson 1991; Krueger & Orme-Johnson 1983). *In vitro* studies have demonstrated that transfection of either MA-10 murine Leydig tumour cells or COS-1 cells with the cDNA of the StAR precursor protein increases the conversion of cholesterol to pregnenelone (Clark *et al* 1994; Sugawara *et al* 1995).

Perhaps the most compelling evidence for the role of StAR in steroidogenesis came when several nonsense mutations within its gene were identified as causing the

disorder, congenital lipoid adrenal hyperplasia (lipoid CAH) (Lin *et al* 1995). Lipoid CAH is a lethal, inherited disorder characterized by severely impaired steroid hormone biosynthesis and enlarged adrenal glands containing high levels of cholesterol and cholesterol esters due to the impaired delivery of cholesterol to P450scc. Targeted disruption of the StAR gene to produce knock-out mice results in a similar phenotype to that of congenital adrenal hyperplasia (Caron *et al* 1997; Hasegawa *et al* 2000).

The mechanism by which StAR regulates cholesterol transfer to the inner mitochondria remains unclear. It was thought that during the import of StAR into the mitochondria, contact sites form between the outer and inner membrane allowing cholesterol import (Stocco & Clark 1996). However, studies using recombinant StAR proteins lacking the mitochondrial import sequence have biological activity equivalent to wildtype StAR, demonstrating that mitochondrial import is not essential for activity (Arakane *et al* 1998; Bose *et al* 2002). Moreover, adrenocortical mitochondria have characteristically tubular cristae, which may not form such contact sites.

1.8.3 Cholesterol side-chain cleavage enzyme (P450scc)

This first step in the synthesis of all steroids is the cleavage of the side-chain of cholesterol by the cholesterol side-chain cleavage enzyme (P450scc). It is located on the inner mitochondrial membrane and catalyzes the conversion of C27 cholesterol to C21 pregnenolone by 3 separate reactions: 20α-hydroxylation, 22α-hydroxylation and a C20-22 bond scission. The location of this enzyme highlighted the mitochondria as an important site for cholesterol delivery. The *CYP11A1* gene located on chromosome 15 encodes for P450scc.

1.8.4 3β Hydroxysteroid dehydrogenase (3β-HSD)

The product of side chain cleavage of cholesterol, pregnenolone, exits the mitochondria and is converted within the cytoplasm by the enzyme 3β-HSD to form progesterone. 3β-HSD also converts 17-hydroxypregnenolone, which is formed from pregnenolone by *CYP17* (see **section 1.8.5**), to 17-hydroxyprogesterone, and dehydroepiandrosterone (DHEA) to androstenedione. 3β-HSD is a member of the microsomal short chain dehydrogenase family and the only non-P450 enzyme in the pathway. Two isoenzymes have been identified in the human (3β-HSD1 and 2). The isoforms are encoded by separate genes that lie in tandem on chromosome 1, *3β-HSD1* and *3β-HSD 2* respectively (Berube *et al* 1989; Lachance *et al* 1991). It is the 3β-HSD2 isoform that is found in the adrenals and gonads (Lachance *et al* 1991).

1.8.5 17α-Hydroxylase(P450c17)

Progesterone and pregnenolone are both 'hydroxylated' by the enzyme 17α-hydroxylase (P450c17), which is encoded by the *CYP17* gene on chromosome 10, to produce 17OH-progesterone and 17OH-pregnenolone respectively (Sparkes *et al* 1991). This 17α-hydroxylation step is essential for glucocorticoid, androgen and oestrogen synthesis. 17OH-Progesterone and 17OH-pregnenolone are converted by a 17,20 lyase reaction catalyzed by the same enzyme to form the adrenal androgens, androstenedione and dehydroepiandrosterone (DHEA) respectively. The zona glomerulosa, where pregnenolone is converted to produce the mineralocorticoid aldosterone, does not express P450c17. Therefore, P450c17 is important in determining the zone specific nature of adrenal steroid synthesis.

1.8.6 21-Hydroxylase (P450c21)

The enzyme 21-hydroxylase (P450c21) is encoded by the *CYP21* gene on chromosome 6 (White *et al* 1986). P450c21 performs 21-hydroxylations to produce 11-deoxycorticosterone (DOC) or 11-deoxycortisol (S) from either progesterone (zona glomerulosa) or 17-OH progesterone (zona fasciculata) respectively. P450c21 is expressed in all three zones of the adrenal cortex (Shinzawa *et al* 1988).

1.8.7 Aldosterone Synthase ($P450_{aldo}$)

The final steps in the synthesis of mineralocorticoids (aldosterone) and glucocorticoids (cortisol) were originally thought to be performed by a single enzyme encoded by a single gene locus (Miller 1987). This is indeed the case of the bovine, porcine and frog enzyme (Nonaka *et al* 1991; Ogishima *et al* 1989). However in man, rat and mouse the final stages are performed by two distinct enzymes. The three terminal stages in the synthesis of aldosterone (11-hydroxylation, 18-hydroxylation and dehydration) are all carried out by the enzyme aldosterone synthase ($P450_{aldo}$), which is encoded by the *CYP11B2* gene on chromosome 8 (Curnow *et al* 1991; Kawamoto *et al* 1992; Mornet *et al* 1989). $P450_{aldo}$ is expressed exclusively in the zona glomerulosa of the adrenal cortex where it performs the following reactions:

1. 11β-hydroxylation of DOC to form corticosterone
2. 18-hydroxylation of B, producing 18-hydroxycorticosterone (18-OHB)
3. 18-oxidation of 18-OHB, resulting in aldosterone

In vitro studies have demonstrated that DOC is the preferred substrate of $P450_{aldo}$ and that it remains bound to the active site throughout these reactions with B and 18-OHB only being released as by-products (Denner *et al* 1995).

1.8.8 11β-hydroxylase ($P450_{11\beta}$)

The final stage in the synthesis of cortisol (or corticosterone in the rat) is an 11-hydroxylation by the enzyme 11β-hydroxylase ($P450_{11\beta}$) encoded by the *CYP11B1* gene on chromosome 8 (or 7 in the rat) (Mornet *et al* 1989). This converts 11-deoxycortisol to cortisol within the mitochondria and is specific to the zona fasciculata-reticularis (Ogishima *et al* 1992). $P450_{11\beta}$ can also convert DOC to corticosterone (B) highlighting the functional similarity with aldosterone synthase. $P450_{11\beta}$ can also perform 18- and 19- hydroxylations of DOC, resulting in 18-OHDOC (18-hydroxy-11-deoxycorticosterone) and 19-OHDOC (19-hydroxy-11-deoxycorticosterone), respectively as well as the conversion of corticosterone, to 18-OHB (18-hydroxycorticosterone) (Okamoto & Nonaka 1992).

1.9 Effects of Corticosteroids

Corticosteroids are involved in a wide range of physiological systems. Some vital functions of glucocorticoids include the regulation of carbohydrate, protein and lipid metabolism, inflammatory, immunogenic and stress responses and the maintenance of blood pressure and cardiovascular function. Mineralocorticoids are essential for the control of electrolyte and water balance. Two homologous corticosteroid receptors have been identified, the mineralocorticoid receptor (MR) (Arriza *et al* 1987) and the glucocorticoid receptor (GR) (Hollenberg *et al* 1985). The MR and GR receptors both belong to the thyroid/steroid hormone receptor superfamily of

transcription factors and share 94% amino acid conservation within their central DNA binding domain (DBD) and approximately 57% within their C-terminal ligand-binding domain (Arriza *et al* 1987). Of particular interest here are the ways in which corticosteroids influence blood pressure.

1.9.1 Mineralocorticoid Effects

The term 'mineralocorticoid' is derived from the first recognized and the physiologically most important action of aldosterone, to regulate transport of electrolytes across epithelial surfaces. The main site of action is the kidney but the salivary glands and the colon also respond (Orth *et al* 1992). Recent work has suggested that aldosterone also exerts rapid non-genomic effects via membrane aldosterone receptors that are distinct from its effects on gene transcription (see **section 1.9.1.5**).

1.9.1.1 Epithelial actions of aldosterone

Aldosterone exerts its classic genomic effects on epithelia by binding to the MR within the cytosol. MR has high affinity for aldosterone, corticosterone and cortisol but is specifically activated by aldosterone in aldosterone target tissues due to the inactivation of the glucocorticoids by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) (see **section 1.9.3**). In the absence of ligand, MR is maintained in an inactive state by association with a complex of chaperone proteins including hsp90. Upon ligand-binding, these proteins dissociate revealing nuclear localization signals and allowing translocation of the ligand-receptor complex to the nucleus (see **figure 1.6**). There it acts as a transcription factor by binding to a nuclear hormone response element (HRE) (with the consensus sequence AGAACAnnnTGTTCT) in

the 5'UTR of aldosterone-responsive genes. This results in activation or repression of expression of specific target genes. Hormone-receptor complexes bind to DNA as dimers. More recently it has been demonstrated that activated steroid receptors can also affect transcription independent of DNA binding by direct protein-protein interactions with other factors (Karin 1998; Reichardt *et al* 1998) (see **section 1.9.2**).

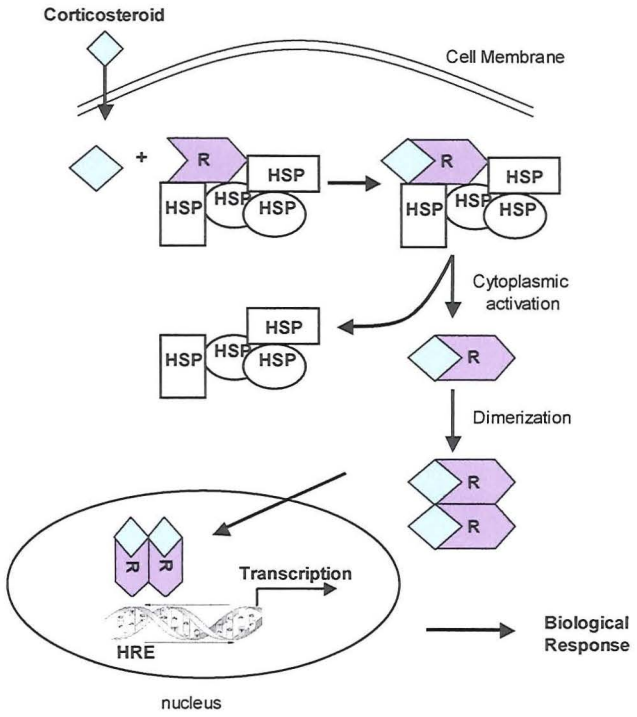


Figure 1.6. Ligand mediated activation of the corticosteroid receptor (MR or GR). *(Adapted from Weigel 1996)*

In the absence of hormone, each receptor (R) monomer is associated with a heat-shock protein complex (HSP). Binding of steroid causes a conformational change resulting in dissociation of the heat-shock complex and allowing dimerization of the receptor and DNA binding to a hormone response element (HRE) to alter transcription of target genes.

The net effect of aldosterone within epithelia is to increase the reabsorption of Na^+ ions at the expense of K^+ and H^+ ions (White 1994) (see **figure 1.7**). The principal sites of aldosterone action within the kidney are the luminal cells of the distal convoluted tubule and the collecting duct as reflected by the high expression levels

of MR at these sites. Aldosterone-responsive epithelia have high electrical resistance and regulated permeability to water and electrolytes. Sodium reabsorption at the apical membrane is facilitated by an electrochemical gradient and mediated by the amiloride-sensitive epithelial sodium channel (ENaC). The ENaC is the rate-limiting step and major determinant of renal sodium reabsorption (Horisberger 1998). Sodium transport out of the cell is by means of active transport across the basolateral membrane mediated by the sodium-potassium ATPase (Na^+-K^+-ATPase). This drives the entry of sodium coupled to the excretion of potassium via the luminal K^+ channel. Sodium reabsorption is accompanied by an influx of water. In addition to these main targets, other potential mediators of aldosterone action are the luminal Na^+/H^+-exchanger (NHE3) in the colon and the luminal thiazide-sensitive Na^+/Cl^- cotransporter in the distal renal tubule (Cho *et al* 1998; Kim *et al* 1998). The principal mechanism of aldosterone-induced electrolyte transport appears to involve an increased activity of ENaC, the limiting step in the transport process. Evidence suggests that aldosterone regulates ENaC activity by increasing the number of channels at the apical membrane and/or by increasing their open-probability (Garty & Palmer 1997; Verrey 1999). The resulting gene products are thought to mediate these effects and are termed aldosterone-induced protein(s) (AIP).

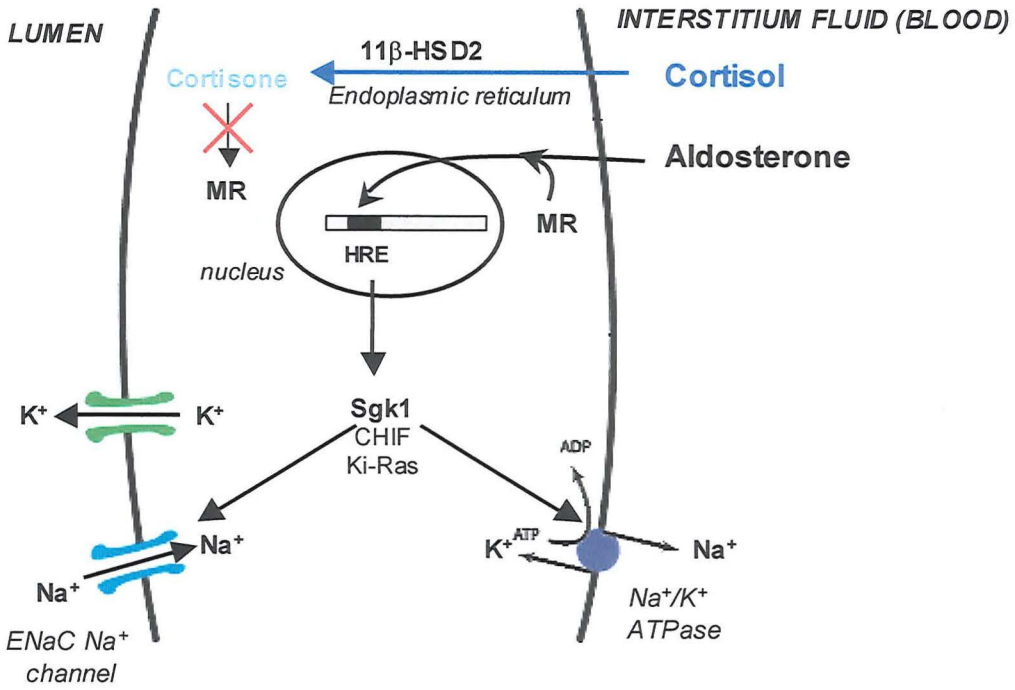


Figure 1.7. The classical genomic action of aldosterone on epithelial tissue.
HRE – hormone response element
(Adapted from Connell & Davies 2005)

1.9.1.2 Aldosterone-Induced Proteins

AIP's may exert effects on the apical membrane, the basolateral Na^+-K^+-ATPase pump and/or cellular energy production to produce an increase in sodium reabsorption with a concomitant potassium and hydrogen ion excretion. Several putative AIP's have been identified.

Sgk1

The serum- and glucocorticoid-regulated kinase 1 (sgk1) was one of the first identified and perhaps is the best characterized of the AIPs. Aldosterone increases

expression of sgk1 in the kidney and the distal colon *in vitro* and *in vivo*. Coexpression studies of sgk1 with the ENaC in *Xenopus larvis* oocytes demonstrate strong activation of the ENaC-mediated Na^+ transport by sgk1; it also appears to activate the Na^+/K^+-ATPase and $Na^+/K^+/Cl^-$ cotransporter (Henke *et al* 2002; Naray-Fejes-Toth *et al* 1999; Shigaev *et al* 2000). There is some evidence that sgk1 may phosphorylate the ENaC α-subunit to alter channel activity or that it can regulate the gene expression of the subunits but the precise mechanism of action of sgk1 remains unclear (Boyd & Naray-Fejes-Toth 2005; Diakov & Korbmacher 2004). However, sgk1 modulates the activity of the ENaC regulator protein Nedd4-2 (neuronal precursor cell-expressed, developmentally down regulated protein 4-2). The importance of this protein in the regulation of ENaC is highlighted by the pathology of Liddle's syndrome. Nedd4-2 is a ubiquitin-protein ligase that regulates the degradation rate of ENaC. Sgk1 kinase interacts with Nedd4-2 and modulates its activity by phosphorylation to reduce its binding to ENaC (Debonneville *et al* 2001; Synder *et al* 2002). This results in a reduced ubiquitination of ENaC, with a subsequent increase in channel density and stability at the apical membrane and an increased capacity for sodium reabsorption. The sgk1-knockout mouse has symptoms of pseuedohypoaldosteronism, similar to that of the MR knockout (Berger *et al* 1998; Wulff *et al* 2002). However, the phenotype in the MR knockout is much more severe, suggesting there are other essential targets of MR action.

Corticosteroid hormone-induced factor (CHIF)

Corticosteroid hormone-induced factor (CHIF) belongs to the FXYD family of small transmembrane proteins that regulate the activity of ion channels and transport proteins. The γ-subunit of Na^+/K^+-ATPase also belongs to this family and shares

>50% sequence homology with CHIF (Geering *et al* 2003). CHIF is expressed within the kidney and distal colon on the basolateral epithelial membranes. In the distal colon, aldosterone, dexamethasone, Na^+ restriction and K^+ loading have all been shown to increase the expression of CHIF (Brennan & Fuller 1999; Capurro *et al* 1996). Na^+ restriction increases CHIF protein levels in the kidney and the colon (Shi *et al* 2001). The precise physiological function of CHIF is unknown, but it is associated with the Na^+/K^+-ATPase in renal tissue and modulates its tranport properties by enhancing the affinity of the Na^+/K^+-ATPase for Na^+ (Beguin *et al* 2001). This may at least partly explain the increase in Na^+/K^+-ATPase activity in response to aldosterone.

Kirsten Ras GTP-binding protein-2A (Ki-RasA)

Ki-RasA is a small monomeric GTP-binding protein that appears to play an important role in mediating aldosterone action in renal epithelia. Aldosterone increases Ki-RasA expression and protein levels during the early phase of action, with increases as early as 30 minutes after aldosterone administration. There is some controversy surrounding the importance of Ki-RasA to aldosterone action as the levels of Ki-Ras or its induction by aldosterone appears to be very small (or absent) in some of aldosterone's target epithelia. Evidence suggests that Ki-RasA increases the open probability of the ENaC channel but decreases the number of channels. The mechanisms for these apparently conflicting actions remain unclear (Spindler & Verrey 1999; Stockand 2002; Stockand & Meszaros 2003).

PI3K

The enzyme, phosphatidylinositide 3-kinase (PI3K), phosphorylates inositol phospholipids to produce the second messengers, 3-phosphorylated inositides, which

mediate diverse cellular signalling functions. PI3K is not an AIP but aldosterone increases its activity, perhaps through induction of Ki-RasA. Inhibition of PI3K affects both the early and late phases of aldosterone action, with a decrease in Na^+ transport even in the presence of sustained aldosterone levels. PI3K also appears to be important for the sodium transport changes mediated by insulin and vasopressin, indicating a point of convergence for the signalling pathways of these hormones to interact and regulate ENaC and Na^+/K^+-ATPase. (Blazer-Yost *et al* 1999; Stockand 2002).

1.9.1.3 Nonepithelial aldosterone action

As well as the principal site of aldosterone action on epithelial cells surfaces, MR have also been identified in a number of nonepithelial cells including vascular smooth muscle and endothelial cells (Christ *et al* 1995; Takeda *et al* 1996), neurones and support cells (Qin *et al* 2003), circulating lymphocytes (Wehling *et al* 1990), cardiomyocytes (Stockand & Meszaros 2003; Wang *et al* 2002) and cardiac fibroblasts (Stockand & Meszaros 2003). It is now accepted that aldosterone has physiological and pathophysiological effects in nonepithelial tissues, especially in the central nervous system (CNS) and cardiovascular system. Of particular interest to this study are the effects on the cardiovascular system.

1.9.1.4 Cardiovascular effects of aldosterone

Within the cardiovascular system, aldosterone is thought to exert effects on both the vasculature and the heart, causing vascular inflammation and damage as well as cardiac hypertrophy and fibrosis, by pathways independent of its effects on blood pressure. However, all these changes may themselves contribute to an altered blood pressure regulation.

Aldosterone effects on the vasculature

In the vascular system, aldosterone promotes smooth muscle hypertrophy and endothelial dysfunction. Activation of MR in vascular smooth muscle cells increases the contractile response to adrenergic stimulation and impairs the vasodilatory response to acetylcholine (Farquharson & Struthers 2002; Jazayeri & Meyer, III 1989; Kornel *et al* 1988; Taddei *et al* 1993). Aldosterone also stimulates an increase in the Ang II receptor (AT1) density in cardiovascular tissues, which may also modulate vascular tone (Robert *et al* 1999). Aldosterone decreases the availability of the vasodilator nitric oxide (NO) and enhances vascular and systemic oxidative stress. These effects are thought to contribute to the vascular alterations observed in the spontaneously hypertensive rat (Sanz-Rosa *et al* 2005). In patients with heart failure spironolactone, a non-specific MR antagonist (which also acts on androgen receptors), greatly increases NO bioactivity, with a concomitant improvement in endothelial function (Farquharson & Struthers 2000). Excess aldosterone is also thought to cause vascular remodelling through collagen deposition in the blood vessels with a resulting decrease in compliance. In support of this, there is evidence to suggest that aldosterone levels are inversely related to arterial compliance in essential hypertension (Blacher *et al* 1997).

Aldosterone effects on the heart

Aldosterone promotes cardiac hypertrophy as well as perivascular and interstitial fibrosis (Brilla *et al* 1990). Hypertension is known to cause cardiac hypertrophy and fibrosis through hemodynamic changes, but the aldosterone-induced hypertrophy and fibrosis, although dependent on salt status, have been shown to occur independently of blood pressure. In support of this, patients with primary aldosteronism are more

likely to have left ventricular hypertrophy (LVH) or stroke than in age and sex matched subjects with essential hypertension of similar severity (Rossi *et al* 1997; Tanabe *et al* 1997).

The importance of MR activation in promoting cardiovascular injury has been recently further highlighted by several clinical trials, including The Randomized Aldactone Evaluation Study (RALES) (Pitt *et al* 1999) and the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) (Pitt *et al* 2003). Addition of low doses of MR antagonist to standard therapy reduced cardiac moribidity and mortality in heart failure patients and in patients after an acute myocardial infarction. This effect was independent of blood pressure reduction or fluid loss, suggesting that antagonism of the non-classical actions of aldosterone at non-epithelial sites, such as the heart and vasculature, may be responsible for the beneficial effects. A substudy of RALES demonstrated that markers of collagen synthesis, which associate with cardiac fibrosis, correlated with disease severity and increased risk. Low doses of spironolactone decreased the levels of collagen, with an associated decrease in risk. This supports a role for aldosterone and cardiac fibrosis in the pathology of heart failure (Zannad *et al* 2000).

The mechanisms of these aldosterone-induced effects is unclear but are thought to involve the regulation of collagen deposition in cardiac tissue (Brilla *et al* 1990). In the presence of a high sodium intake, aldosterone exerts a direct pro-fibrotic action by stimulating the accumulation of fibrillar type 1 collagen in the myocardium with associated hypertension and LVH (Brilla & Weber 1992).

Oxidative stress and perivascular inflammation appear to be indirect mediators of aldosterone-induced fibrosis. After several weeks, aldosterone/salt treatment of rats induces coronary inflammation, characterized by macrophage, monocyte and fibroblast inflitration of the perivascular space and increases in mRNA for pro-inflammatory mediators and cytokines (Farquharson & Struthers 2002; Rocha & Funder 2002). This inflammatory response appears to be necessary for the accumulation of fibrillar collagen and fibrous tissue within the heart.

Aldosterone also increases the expression of NADPH oxidase, which in turn catalyses the formation of the superoxide anion. The superoxide anion reacts with NO to form peroxynitrite, reducing NO bioavailability with a resulting proinflammatory and fibrogenic phenotype. All these responses can be blocked by MR antagonists or antioxidant agents such as N-acetylcysteine, suggesting that oxidative stress is at least partly responsible for the pro-inflammatory phenotype induced by aldosterone (Rocha & Funder 2002; Sun *et al* 2002).

A role for the classic MR in mediating these cardiac effects is suggested as most of the observed phenotypes take several weeks to develop and the antagonist spironolactone is able to prevent aldosterone-induced fibrosis (Brilla *et al* 1993). It has recently been demonstrated that the vascular inflammation and cardiac fibrosis stimulated in rats by 4 weeks of DOC treatment, can be completely reversed by the eplerenone but not mineralocorticoid withdrawal alone (Young & Funder 2004). MRs have been detected in all four chambers of the heart, within cardiac myocytes, endocardium, VSMC and endothelial cells (Pearce & Funder 1987). However, whether the MR protector 11βHSD2 is expressed here remains controversial. If expression occurs, it may only be at low levels and within specific cell types (eg.

VSMC) (Funder 2005). This implies that within the heart, MR is essentially a high affinity GR due to the much higher circulating levels of cortisol. This effect would be further enhanced if 11βHSD1 (converting cortisone to cortisol) is expressed. It has been identified *in vitro* in cardiac fibroblasts (Sheppard & Autelitano 2002). It has been suggested that local production of aldosterone in the heart could generate high local intracardiac levels, enabling MR activation by aldosterone but the lack of cardiac remodelling in adrenalectomized rats suggests this is unlikely (Rocha & Funder 2002). However, co-infusion with corticosterone antagonizes the actions of aldosterone in the heart, preventing a rise in blood pressure and cardiac fibrosis. Glucocorticoids may have a protective role within the heart (Young & Funder 1996).

1.9.1.5 Non-genomic actions of aldosterone

In addition to the classic genomic mechanism of aldosterone action, there is also increasing evidence that aldosterone can also exert rapid, non-genomic effects that involve the activation of second messenger pathways (Wehling *et al* 1992; Wehling 1995). The genomic effects mediated through the MR involve steroid-receptor regulation of gene expression and involve a latent period of 1-2 hours. These genomic effects can be blocked by inhibitors of transcription (actinomycin D) or translation (cycloheximide) as well as by MR antagonists such as spironolactone. Several recent studies have reported rapid effects that occur within 15 minutes. These are believed to be independent of gene transcription and translation as they are not blocked by actinomycin D or cycloheximide (Falkenstein *et al* 2000; Harvey *et al* 2001). These rapid effects have been predominantly demonstrated outwith the kidney in cells such as vascular smooth muscle cells (Christ *et al* 1995) skeletal muscle cells (Passaquin *et al* 1998) and myocardial cells (Barbato *et al* 2002). A

rapid (within 4 minutes) vasoconstrictor response to a modest infusion of aldosterone has been recently demonstrated *in vivo* (Romagni *et al* 2003). Whether these effects are mediated through the classic MR or a novel non-genomic receptor remains controversial. Wehling et al demonstrated a rapid response (1-2mins) to aldosterone on the Na^+/H^+-exchanger and inositoltrisphosphate generation in human lymphocytes and vascular smooth muscle cells. This effect was not mediated by cortisol and was not blocked by the MR antagonist spironolactone, supporting the existence of a distinct, novel receptor (Wehling 1995). A similar pattern was observed in kidney cells where aldosterone induced a rapid rise in cytosolic calcium that could not be blocked by the classic MR antagonists (Gekle *et al* 1997). This rapid response of calcium to aldosterone can also be induced in MR knockout mice, further supporting the possibility of distinct receptors for rapid signalling (Haseroth *et al* 1999). However, in vascular tissue the rapid nongenomic effect of aldosterone on intracellular Ca^{2+} levels, the Na^+/H^+ exchanger and vasoconstriction are blocked by the MR antagonist eplerenone, suggesting a role for the classic MR (Michea *et al* 2005). In rat smooth muscle cells, the rapid, aldosterone-mediated sodium efflux, which is not blocked by actinomycin, also appears to be MR-dependent. However, the recent identification of membrane bound G-protein-coupled receptors for progesterone and oestrogen supports the possibility that a similar receptor exists for aldosterone. A candidate aldosterone membrane receptor of 50kDa was isolated from human lymphocytes using binding assays but the complete structural characteristics are yet to be determined (Eisen *et al* 1994).

These nongenomic effects of aldosterone (Wehling *et al* 1998) have been linked to the development of increased systemic vascular resistance and so could contribute to hypertension and cardiovascular disease (Armanini *et al* 1991).

1.9.2 Glucocorticoid Effects

Cortisol has a wide range of effects throughout many tissues (brain, heart, blood vessels and kidney) (Sapolsky *et al* 2006) as indicated by the almost ubiquitous expression of its receptor (GR) (Ballard *et al* 1974). Glucocorticoids are known to acutely increase blood pressure and long-term administration has been shown to induce hypertension in humans (Whitworth *et al* 2000). The precise mechanisms by which cortisol regulates blood pressure are complex and not fully understood but appear to involve several actions on the vasculature and kidney. Cortisol administration results in an increased pressor responsiveness of the vasculature to catecholamines and to a lesser extent Ang II (Whitworth 1994). This may at least partly occur through upregulation of the receptors for these vasoconstrictors, including AT1A. There is also increasing evidence that cortisol inhibits the response of the vasodilator, nitric oxide, by inhibiting the enzyme nitric oxide synthase (NOS), by preventing transport of the precursor arginine or by inhibiting the synthesis of the NOS cofactor, tetrahydrobiopterin (Mitchell & Webb 2002; Whitworth *et al* 2000).

Cortisol also has indirect effects on the kidney by stimulating angiotensinogen (AGT) expression in the liver via two glucocorticoid response elements (GRE's) (one full and one half), on the AGT gene. The resulting increase in AGT concentration affects intravascular RAS activity, resulting in Ang II-dependent changes in blood pressure (Braiser & Li 1996). Another possibility is that cortisol activates the MR within the kidney to promote sodium and water retention. Indeed, kinetic properties of the enzyme 11βHSD2 (the MR protector, see **section 1.9.3**) suggest that even high normal physiological cortisol concentrations could saturate

this enzyme (Ferrari 2003). Therefore, cortisol is able to modulate blood pressure by altering both intravascular volume and vascular resistance.

The cytosolic GR receptor is homologous to and functions in a similar way to MR (Gustafsson *et al* 1987). However, GR has moderate affinity for cortisol and corticosterone with low affinity for aldosterone and DOC. Ligand binding induces GR to dissociate from the complex of heat shock proteins that maintain it in a stable state and to form homodimers. This dimerization is thought to occur prior to DNA binding. The GR/steroid dimer complex binds to specific regions within target genes called glucocorticoid-responsive elements (GRE) with the consensus palindromic DNA sequence 5'-GGTACAnnnTGTTCT-3'. There is also evidence that GR can form heterodimers with other transacting factors including MR. The interaction of GR with GRE inititates or represses transcription with the help of various coactivator or corepressor proteins. There is also evidence that GR can exert its effects on gene transcription independent of DNA binding through protein-protein interactions with other factors (Beato & Sanchez-Pacheco 1996; Karin 1998; Reichardt *et al* 1998; Stocklin 1996). The following molecular mechanisms of glucocorticoid/receptor action on gene transcription have been observed (see **figure 1.8**):

1. Trans-activation - Steroid/GR complex binds GRE and increases expression of target genes (Strähle *et al* 1987).

2. Trans-modulation - Steroid/GR complex binds composite GREs comprised of half GRE and a half binding site for another transcription factor (eg. AP-1) – alter transcription of other TF's (Diamond *et al* 1990; Pearce & Yamamoto 1993).

3. Trans-repression - Steroid/GR complex binds negative GRE sequences (nGRE) and repression of transcription of target genes (Beato *et al* 1989).

4. Transcription synergy - Protein-protein interaction with factors such as Stat-5 (Stocklin 1996).

5. Transcription repression - Protein-protein interaction with factors such as AP-1, CREB or NF-κB (De Bosscher *et al* 2003).

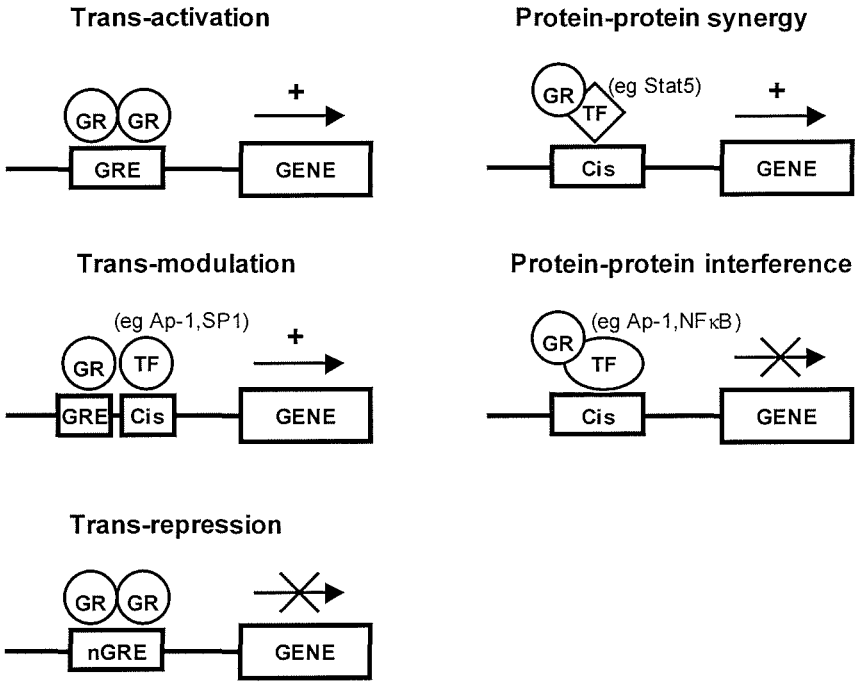


Figure 1.8. Mechanisms of transcriptional regulation by the glucocorticoid receptor
TF = transcription factor
(Adapted from Karin 1998; Reichardt & Schutz 1998)

As mentioned previously, the GR is almost ubiquitously expressed compared to the relatively selective expression of MR. Due to the high degree of homology between MR and GR, it is perhaps not surprising that there is some overlap of ligand binding. In particular, the MR has been shown to have the same affinity for aldosterone, corticosterone and cortisol *in vitro* (Krozowski & Funder 1983). In combination with the much higher circulating levels of cortisol (100 - 1000 fold) compared to aldosterone within the body, a mechanism is clearly required to ensure the specific

activation of MR by aldosterone. This mechanism is described in the following section.

1.9.3 11β-hydroxysteroid Dehydrogenases

The enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) confers specificity on the MR by inactivating cortisol (Edwards *et al* 1988; Funder *et al* 1988) (see **figure 1.9**). This NAD-dependent enzyme is coexpressed with MR in aldosterone target tissues (kidney, colon and salivary glands) and converts cortisol into the inactive 11-keto metabolite, cortisone. Disruption of this enzyme-protective system, due to mutations in the 11β-HSD2 gene, results in the syndrome of apparent mineralocorticoid excess (AME) in which cortisol is free to act like a mineralocorticoid and activate MR (Mune *et al* 1995) (see **section 1.15.2.2**). Another distinct 11β-HSD isoenzyme (11β-HSD1) is expressed mainly in the liver and visceral adipose tissue, as well as the brain, gonads, muscle, lung and other GR-expressing tissues (Moisan *et al* 1990; Monder & White 1993; Walker *et al* 1992) (see **figure 1.9**). This isoform requires NADP(H) which is supplied by hexose-6-phosphate dehydrogenase (H6PDH). 11β-HSD1 can function bidirectionally (Monder & White 1993) but has been shown *in vivo* to act as an 11-oxo-reductase, particularly in hepatocytes and adipocytes, thereby enhancing GR-dependant functions at these sites including gluconeogenesis and adipocyte differentiation (Seckl & Walker 2001). Therefore, inhibition of 11β-HSD1 is a potential target for treatment of disorders that might be ameliorated by local reduction of glucocorticoid action, such as type 11 diabetes and obesity.

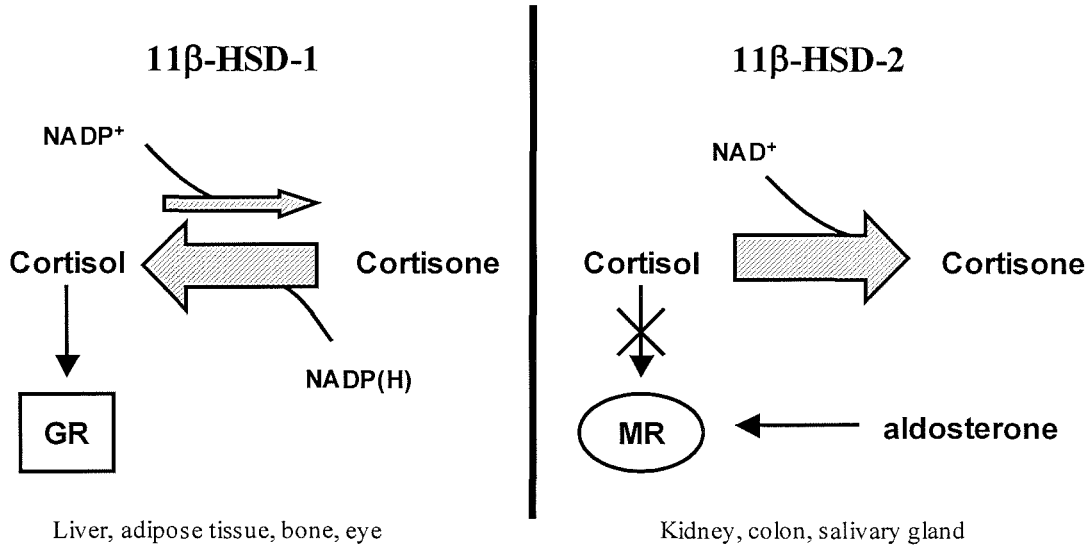


Figure 1.9. Schematic role of 11β-HSD isozymes and their main sites of action *(Adapted from Arlt & Stewart 2005)*

The 11β-HSD enzymes function to provide tissue-specific mineralocorticoid and glucocorticoid effects in the appropriate tissues throughout the body.

1.9.4 Extra-adrenal Corticosteroid Production

Within the vasculature and the brain, all the enzymes required for the de novo synthesis of corticosteroids from cholesterol have been identified (Davies & MacKenzie 2003). Therefore, locally produced aldosterone could act in an autocrine/paracrine manner to exert the non-classical effects mentioned previously. However, the level of transcription to mRNA suggests that local steroid production is small in comparison with adrenal production and so whether extra-adrenal

corticosteroids are of any physiological significance remains unclear. However intracerebroventricular (icv) infusion of aldosterone, at doses far too low to have an effect when administered subcutaneously, significantly increases blood pressure (Gomez-Sanchez 1986). Therefore small local amounts can have an effect. Local concentration, proximity to target cells and, possibly, tissue-specific control mechanisms may all be determining factors. In theory, local aldosterone production in the heart would provide an important therapeutic target for cardiovascular disease, especially considering the suggestion that aldosterone synthase expression is increased in heart failure (Yoshimura *et al* 2002). However, research into local aldosterone production within the heart has yielded contradictory results (Davies & MacKenzie 2003; Funder 2004; Gomez-Sanchez *et al* 2004; Silvestre *et al* 1999; Silvestre *et al* 1998; Young *et al* 2001). There have been reports of a high relative aldosterone concentration in perfused hearts or homogenates (Silvestre *et al* 1999; Silvestre *et al* 1998) and yet recent work using a highly sensitive and specific, quantitative RT-PCR method failed to detect *CYP11B2* expression in several rat models of cardiovascular pathology or in isolated cardiac myocytes treated with angiotensin II and ACTH (Ye *et al* 2005). It is at least possible that the high relative aldosterone concentrations within the heart are due to sequestration of the hormone from the bloodstream or to slower degradation rather than local synthesis. In favour of this, the levels of aldosterone detected in the heart appear to reflect normal circulating plasma levels (Gomez-Sanchez *et al* 2004).

1.10 Abnormal Corticosteroid Production

Abnormalities in corticosteroid production highlight their importance in influencing blood pressure. At a gross level, corticosteroid excess as occurs in Cushing's

syndrome (cortisol excess) or primary aldosteronism (aldosterone excess) are often associated with hypertension whilst Addison's disease (the deficiency of adrenal steroids) is invariably associated with hypotension (White 1994; White & Speiser 1994).

1.10.1 Cortisol Excess

Cushings disease is the result of excess cortisol production due to pituitary adenomas that secrete excess amounts of ACTH. A similar phenotype or syndrome can occur as the result of ectopic ACTH production or adrenal cortisol secreting tumours. The syndrome is characterized by truncal obesity, glucose intolerance and hyperglycaemia, osteoporosis, hyperlipidaemia and hypertension. Hypertension occurs in 70 – 80% of cases. The increased cortisol levels cause saturation of the enzyme 11βHSD2, resulting in cortisol activation of the MR by the kidney. Evidence of impaired 11βHSD2 in patients with Cushings, is demonstrated by the ratio of urinary cortisol:cortisone metabolites (Stewart *et al* 1995; Ulick *et al* 1992a). A similar finding has also been reported in EH (Soro *et al* 1995). However, in subjects with cortisol-induced hypertension, treatment with spironolactone to block the sodium and water retention, have failed to reduce the induced rise in blood pressure suggesting other mechanisms are involved (see **section 1.9.2**)

Excess glucocorticoids, either endogenous (Cushings syndrome) or exogenous (glucocorticoid therapy), can result in hypertension. With cortisol-induced hypertension, the risk of cardiovascular morbidity and mortality is high with an increased incidence of left ventricular hypertrophy compared to other forms of hypertension. The hypertension is associated with an increased cardiac output,

increased renal vascular resistance, increased sodium/water retention and an increased vascular responsiveness.

1.10.2 Aldosterone Excess

Aldosterone excess can occur from rare genetic causes (such as Glucocorticoid suppressible hyperaldosteronism) or primary aldosteronism (PA). PA can be defined as excessive production of aldosterone independent of its normal regulator, Ang II, which is produced by renin (Gordon *et al* 1994a). This relationship is described in detail in **section 1.12.2**. Briefly, autonomous excess of aldosterone will suppress renin levels so that the aldosterone-to-renin ratio (ARR) is elevated. PA is characterized by increased extracellular fluid volume, hypokalaemia, alkalosis and hypertension as a result of the marked sodium and water retention. Jerome Conn first described the symptoms in 1954 as a syndrome related to the hypersecretion of aldosterone by an adrenal adenoma, now known as Conn's syndrome (Conn & Louis 1956). For many years this was thought to be the primary cause of PA, but recent studies suggest that bilateral adrenal hyperplasia (idiopathic hyperaldosteronism) may be a more common cause of PA than previously realised (Lim *et al* 2002a; Montori & Young, Jr. 2002).

1.10.2.1 ARR as a screening tool for PA

Until recently, PA has always been considered to be a rare cause of secondary hypertension, accounting for less than 1% of cases. However, recent use of the ARR as a reliable marker of inappropriate aldosterone activity has led to an increased frequency of the diagnosis of PA among hypertensive patients. Generally an ARR >750 (when aldosterone is expressed in pmol/L and renin as renin activity in

ng/ml/hour) is accepted as higher than the normal range. A number of independent studies worldwide, including Scotland (Lim *et al* 1999b; Lim *et al* 2002a), Australia (Gordon *et al* 1994b), South East Asia (Loh *et al* 2000) and South America (Fardella *et al* 2000), demonstrate the prevalence of PA using a high ARR as a marker to be between 5 to 15% in unselected individuals with hypertension, with a much higher proportion of bilateral adrenal hyperplasia (BAH) (Mulatero *et al* 2004). In most of these studies, less than half of the identified patients were hypokalemic, the previous prerequisite for diagnosis of mineralocorticoid hypertension. The idea that a normokalemic form of PA may account for as many as 20% of patients with essential hypertension was first suggested by Conn in 1965 but has largely been forgotten until now (Conn *et al* 1965).

The ARR measurement itself is not conclusive and further confirmatory tests such as fludrocortisone suppression test or salt loading are required to demonstrate inappropriate regulation of aldosterone levels. Much of the controversy surrounding the use of the ARR as a diagnostic tool for PA is due to the plasma renin activity dominating the ratio. In many cases, the elevated ratio is due to low renin levels and aldosterone levels are often within the 'normal' range and so would not be traditionally diagnosed as PA. It is unclear how these subjects differ from the previously well-defined subgroup of low renin essential hypertension, which is also characterized by aldosterone levels that are inappropriately high for the prevailing renin level. Padfield et al proposed some years ago that patients with PA due to BAH are no different to the subgroup of low renin essential hypertension (Padfield *et al* 1981). Indeed the presence of adrenal nodules with or without hyperplasia that is characteristic of BAH can also occur in essential hypertension, low renin hypertension or even in normotensive subjects (Kaplan 1967) suggesting there may

be substantial overlap between the groups. There is also histological evidence demonstrating that subjects with aldosterone-producing adenomas often have hyperplasia within the rest of the adrenal, suggesting that solitary adenomas arise on the background of an already abnormal gland (Neville & MacKay 1972). Therefore, with the new diagnostic criteria, PA may be considered a subtype of hypertension with dysregulated renin-angiotensin-aldosterone dynamics that may show progression from hypertension with a raised ARR to BAH and finally to classical PA with truly autonomous aldosterone secretion.

There is still much controversy surrounding the use of the ARR as a screening tool for PA and the classification of hypertensive patients. However, the use of the ARR has lead to an increase in the diagnosis of aldosterone-secreting adenomas, many cases of which can be cured completely by surgery. Also, whatever the classification of subjects with a high ARR as BAH, PA or low renin essential hypertension, although the aldosterone levels may not be particularly elevated, they are still innappropriate for the prevailing levels of renin and Ang II. This highlights a role for altered aldosterone production or control as a key phenotype in essential hypertension. In support of this is the observation that patients with a raised ARR respond particularly well to treatment with selective MR antagonists such as spironolactone (Lim *et al* 1999a).

It is clear from the clinical syndromes of Cushing's disease and PA that abnormalities in corticosteroid production can be associated with hypertension. However, to further explore the contribution of corticosteroids to the genetic component of essential hypertension, a review of the molecular genetics of aldosterone and cortisol synthesis is required.

1.11 The *CYP11B1* and *CYP11B2* Genes

As mentioned previously, in man the three terminal stages in the synthesis of aldosterone (11β-hydroxylation, 18-hydroxylation and 18-oxidation) from DOC are all catalyzed by aldosterone synthase (encoded by *CYP11B2*). Cortisol is also synthesized by 11β-hydroxylation, in this case of 11-deoxycortisol, and catalyzed by the enzyme 11β-hydroxylase (encoded by *CYP11B1*). In other species such as bovine, pig and bullfrog, there is only a single *CYP11B1* gene for the synthesis of cortisol and aldosterone (Nonaka *et al* 1995).

CYP11B2 and *CYP11B1* are situated approximately 40 kilobases apart, on chromosome 8 in man. They are both approximately 6.5kb long from the start of transcription to the polyadenylation site and contain 9 exons and 8 introns (see **figure 1.10**). They are 95% homologous in their coding regions with the main differences in their sequences being in their 5'UTR regions that are responsible for the differences in their transcriptional regulation and zonal distribution (Chua *et al* 1987; Mornet *et al* 1989; Zhang & Miller 1996).

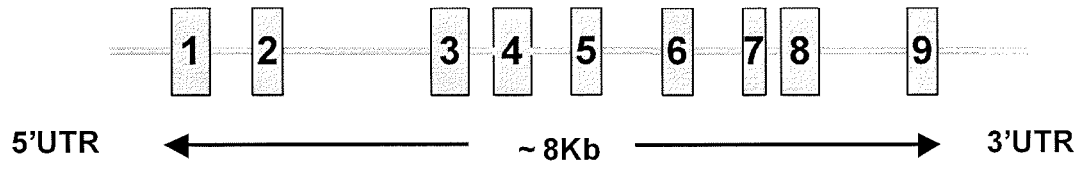


Figure 1.10. Schematic diagram of the exonic-intronic arrangement of the *CYP11B1*/B2 genes

1.12 Regulation of *CYP11B1* and *CYP11B2* Expression

Adrenal steroidogenesis is under both acute and chronic regulation by tropic hormones. The acute response occurs within minutes and involves the mobilization of cholesterol from intracellular stores to the mitochondrial membrane (see **section 1.8.1**) in response to ACTH, Angiotensin II, K^+ and their respective intracellular messenger pathways (see following sections) (Clark *et al* 1995b; Clark *et al* 1995a). Of particular interest to this review is the chronic response that takes several hours and involves the transcription of the genes encoding the steroidogenic enzymes.

1.12.1 ACTH

Corticotrophin releasing hormone (CRH) release is controlled by input from 'higher' centres that respond to physiological and psychological stressors (see **figure 1.11**). CRH is released from the hypothalamus and passes into the hypophyseal portal circulation to stimulate the release of ACTH from the corticotroph cells of the anterior pituitary. ACTH activates its receptor on adrenal cells to stimulate steroidogenesis and increase glucocorticoid (cortisol) synthesis. Glucocorticoid synthesis is controlled by classical endocrine feedback loops whereby cortisol inhibits secretion of CRH from the hypothalamus and ACTH from the pituitary. ACTH is synthesised as part of a large presursor molecule pro-opiomelanocorticotrophin (POMC), which also contains the other peptides melanocyte stimulating hormone (MSH) and β-endorphin.

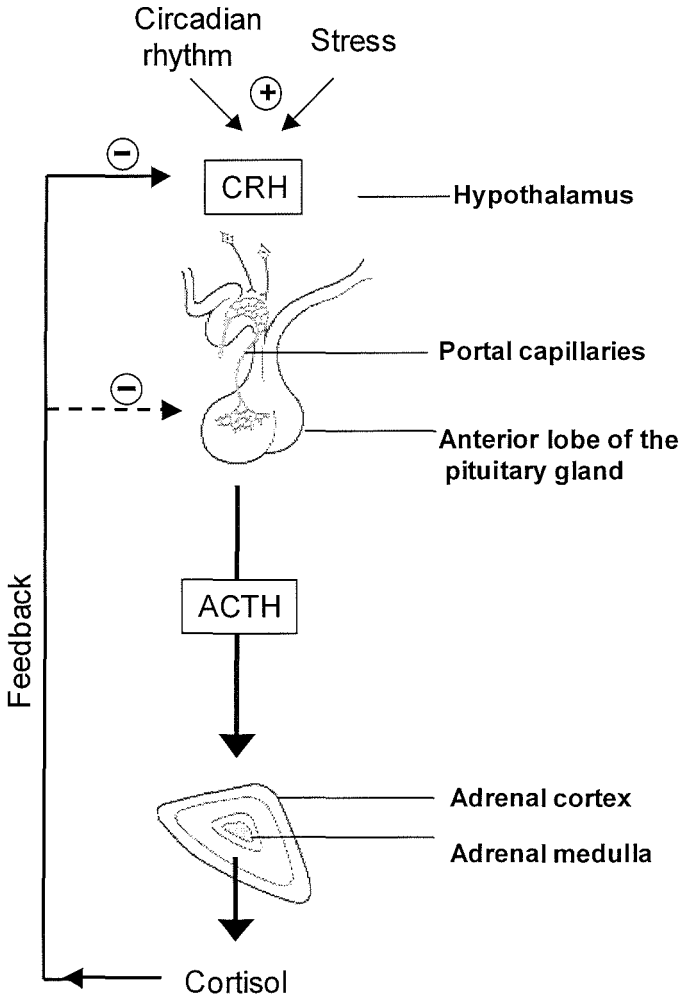


Figure 1.11. Hypothalamic pituitary axis regulation of cortisol secretion

ACTH itself is a 39 amino acid peptide; the first 24 of which are conserved in all species and the first 18 confer its biological activity (Smith & Funder 1988). Normal ACTH release from the anterior pituitary is pulsatile and follows a diurnal rhythm with the highest levels occurring first thing in the morning and the lowest at night (Veldhuis *et al* 1990). ACTH exerts its effects by binding to its receptor (ACTH-R),

a G-protein coupled receptor, which is a member of the melanocortin receptor family (Mountjoy *et al* 1992). After binding to its receptor, ACTH activates adenylate cyclase, resulting in an increased intracellular cAMP concentration and activation of protein kinase A (PKA) (Buckley & Ramachandran 1981; Sala *et al* 1979) (see **figure 1.12**). ACTH plays an essential role in regulating *CYP11B1* gene expression (Sewer & Waterman 2003). Each of the ACTH-responsive genes contain unique cAMP-response sequences (CRS) within their 5' regulatory regions that bind a variety of transcription factors, including homeodomain proteins, SP family, CREB and SF-1 (Waterman & Bischof 1996). ACTH stimulates *CYP11B1* transcription via cAMP and the orphan nuclear receptor, SF-1. The cAMP-mediated increase in steroidogenic gene transcription takes hours and is inhibited by the protein synthesis inhibitor, cycloheximide. This suggests that synthesis of a common, unknown protein factor(s) is required. Several other cAMP-independent intracellular signalling pathways appear to be induced by ACTH, including protein kinase C (PKC) (Arola *et al* 1994), calcium influx via calcium channels (Yamazaki *et al* 1998) and the lipooxygenase pathway (Yamazaki *et al* 1996).

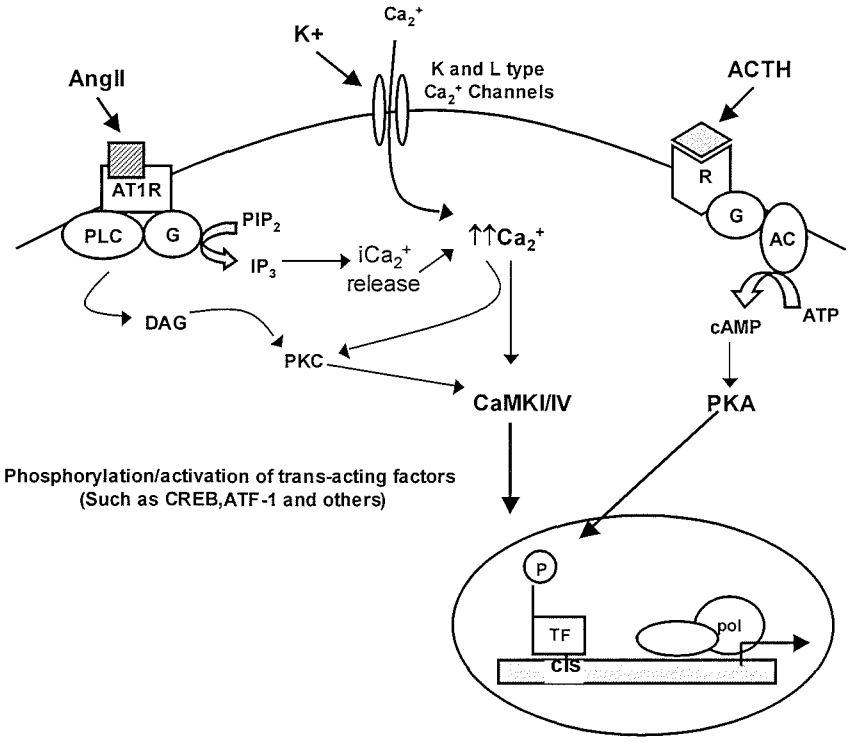


Figure 1.12. Intracellular mechanisms of Ang II, K+ and ACTH influencing gene expression *(Adapted from Cooke 1999; Rossier 1997)*

ACTH has a modest effect on the secretion of aldosterone but the physiological relevence is unclear. ACTH acutely stimulates aldosterone secretion (probably through activation of StAR protein production but in the long-term, has an inhibitory effect on *CYP11B2* gene expression and aldosterone levels. (Aguilera *et al* 1996; Mitani *et al* 1996). It is not completely clear how ACTH inhibits expression of *CYP11B2*. Cyclic AMP (cAMP), the second messenger for ACTH, desensitizes adrenocortical cells to Ang II by causing a reduction in the expression of Ang II receptors (Bird *et al* 1994; Yoshida *et al* 1991). ACTH may also decrease aldosterone production by stimulating the expression of *CYP11B1* and *CYP17*,

resulting in a removal of precursors from the aldosterone pathway and using them to synthesize cortisol. ACTH receptors are present in both the zona glomerulosa and fasciculata. Under normal circulating ACTH levels, the glomerulosa maintains *CYP11B2* expression by at least two mechanisms. Firstly, Ang II will inhibit ACTH-stimulated cAMP production in glomerulosa but not fasciculata cells (Begeot *et al* 1988; Hausdorff *et al* 1987). Secondly, the glomerulosa expresses a type of adenylyl cyclase that is inhibited by increasing intracellular Ca^{2+}, the second messenger for both Ang II and K^+ (Shen *et al* 1997).

Long-term ACTH exposure stimulates adrenal growth both *in vitro* and *in vivo* through promoting hypervascularization as well as cellular hypertrophy and hyperplasia (Arola *et al* 1993; Bornstein *et al* 1992; Bornstein & Chrousos 1999; Vinson 2003). This results in an increase in the number of cells with the potential for steroidogenesis (Arola *et al* 1993; Dallman *et al* 1980). ACTH appears to specifically induce the proliferation of zona fasciculata cells whilst recruiting and transforming glomerulosa cells into fasciculata-like cells (Aguilera *et al* 1981; Arola *et al* 1993; Mazzocchi *et al* 1986)

1.12.2 Angiotensin II and Potassium

Ang II and circulating potassium concentration are the most influential regulators of aldosterone secretion. They act through common intracellular signalling pathways to increase transcription of *CYP11B2* in the zona glomerulosa.

Ang II is an octapeptide hormone generated by the renin-angiotensin-system (RAS) (see **figure 1.13**). The rate-limiting step in the RAS is the secretion of the protease renin from the kidney, which is controlled through a negative feedback loop. Renin

is released from the juxtaglomerular cells in the renal tubule in response to a decrease in renal perfusion pressure and filtrate sodium content and an increased sympathetic tone. It converts angiotensinogen, which is constitutively secreted by the liver, into angiotensin I (Ang I). Ang I, which has no apparent activity, is then converted by angiotensin-converting-enzyme (ACE) into Ang II, the active component of the RAS. Ang II causes an elevation in blood pressure by directly constricting blood vessels, increasing sympathetic nerve activity, increasing myocardial contractility, enhancing renal salt and water retention both directly and indirectly through stimulating aldosterone release from the adrenal (Carpenter *et al* 1961; Gavras *et al* 1976). The resulting increase in Na^+ and water retention as well as blood pressure inhibit renin release. Ang II also has a proliferative effect on tissues, inducing cardiac and vascular hypertrophy, partly through the stimulation of growth factors and cytokines (McConnaughey *et al* 1999). Ang II has been shown to activate NADPH oxidase, resulting in increased production of the superoxide anion ($\cdot O_2^-$), which can react with nitric oxide (NO) to produce the harmful oxidant peroxynitrite ($ONOO^-$). This may contribute to the vasoconstrictive response to Ang II by decreasing the availability of the vasodilator NO (Rajagopalan *et al* 1996).

There are two main receptors for Ang II, AT1 and AT2. AT1 occurs as two subtypes AT1A and AT1B (Kambayashi *et al* 1993; Mukoyama *et al* 1993). Most of the known biological actions of Ang II are mediated through AT1, in particular AT1A, except for the control of aldosterone secretion from the adrenal cortex, which appears to be mediated by AT1B (Gigante *et al* 1997). The AT1 receptor is a G-protein-coupled receptor (GPCR) that exhibits zonal distribution within the adrenal cortex.

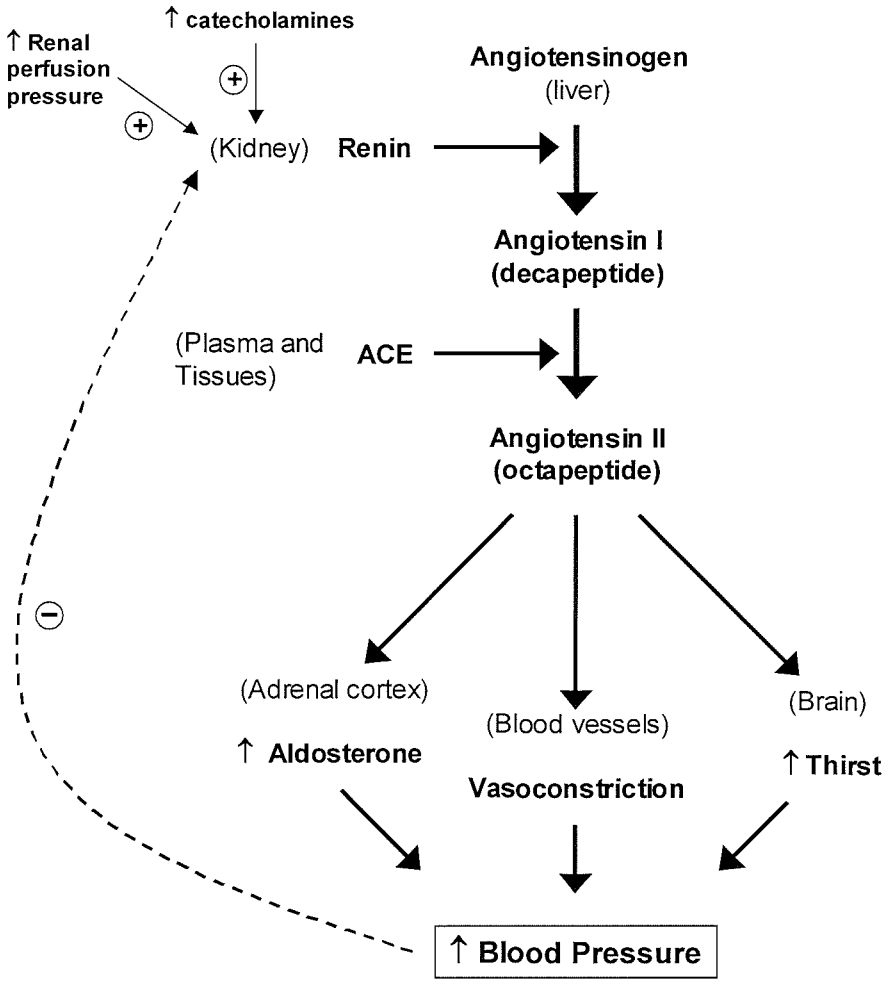


Figure 1.13. Renin-Angiotensin System

Ang II is thought to stimulate aldosterone synthesis through various intracellular signalling pathways including the src family of tyrosine kinases (Sirianni *et al* 2001) and the 12-lipoxygenase (12-LO) pathway (Gu *et al* 2003). However, the best-characterized pathway is the activation of phopholipase C (see **figure 1.12**). Once activated, phospholipase C hydrolyses phosphatidyl inositol 4,5-biphosphate (PIP2) to 1,4,5 inositoltriphosphate (IP3) and diacylglycerol (DAG) resulting in release of Ca^{2+} from intracellular stores and activation of protein kinase C (PKC) respectively. At the same time, the membrane's potassium permeability decreases resulting in

depolarization of the cell and stimulation of voltage-gated calcium channels, causing entry of extracellular calcium (Ca^{2+}). The increased intracellular Ca^{2+} concentration activates calmodulin and regulates *CYP11B2* transcription through various calmodulin-dependent kinases (Condon *et al* 2002). PKC has no apparent effect on *CYP11B2* transcript levels but does inhibit expression of *CYP17* (Bird *et al* 1996), perhaps contributing to the zonation of the adrenal cortex.

The RAS is very sensitive to changes in circulating potassium levels. The level of potassium affects renin secretion as well as having a direct effect on the adrenal cortex to increase aldosterone secretion. Potassium (like Ang II) stimulates aldosterone secretion by increasing expression of *CYP11B2* through an increase in intracellular Ca^{2+} and activation of calmodulin kinases. Potassium signalling is mediated through membrane depolarization, leading to an influx of calcium through T and L-type channels (see **figure 1.12**). K^+ and partially Ang II stimulation of *CYP11B2* mRNA can be blocked by calcium channel blockers such as nifedipine.

1.13 Transcriptional regulation of the *CYP11B* Genes

As mentioned previously, chronic regulation of steroidogenesis involves transcription of the genes encoding the necessary steroidogenic enzymes. This is mediated by alteration of *trans*-acting factors that bind to the *cis*-regulatory elements within the 5' regulatory regions of the target genes.

Investigation of the 5' regulatory regions of the bovine *CYP11B* gene revealed six cis-acting elements (Ad1-6) (Kirita *et al* 1990) (see **figure 1.14**). Deletion and mutation analysis confirmed that Ad1 and a region containing Ad3 and Ad4 were essential for the ACTH/cAMP-induced expression of the gene (Hashimoto *et al*

1992; Kirita *et al* 1990; Takayama *et al* 1994). These sites are highly conserved within the *CYP11B* genes of all species studied so far, including h*CYP11B1* (Wang *et al* 2000) and h*CYP11B2* (Clyne *et al* 1997), with the exception of the rat *CYP11B1* gene (Rainey 1999).

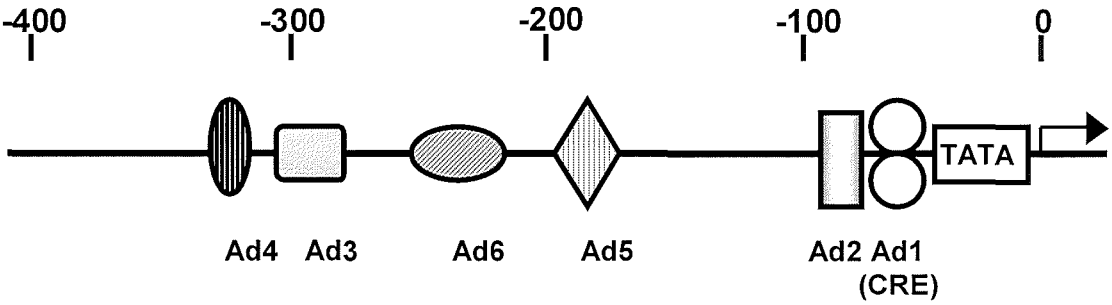


Figure 1.14. Schematic diagram of the bovine *CYP11B* promoter with the cis-elements (Ad1 to Ad6) that are conserved among all the *CYP11B* genes *(Morohashi et al 1993)*

1.13.1 Ad1 (CRE)

The Ad1 element closely resembles a consensus cAMP regulatory element (CRE) site. CRE's play an essential role in cAMP-dependent gene expression of a wide variety of genes. Proteins, such as the CRE-binding protein (CREB) (Montminy & Bilezikjian 1987) and the highly related activating transcription factors (ATF) (Hai *et al* 1989; Hurst *et al* 1990), bind to CRE sites to initiate transcription. CREB binds to DNA as a dimer (Yamamoto *et al* 1988) and has a conserved region of leucine residues (leucine zipper) at its C terminus that enables dimerization and sequence-specific DNA binding (Dwarki *et al* 1990; Yun *et al* 1990). CREB binds to DNA through a domain of basic residues near the leucine zipper. These regions are characteristic of the bZIP family of transcription factors and are highly conserved

between CREB and ATF1 (Rehfuss *et al* 1991). CREB functions as a component of a variety of signalling pathways, particularly PKA (Montminy 1997), but also mitogen-activated protein kinases (MAPKs) (Xing *et al* 1996) and CaMKs (Sheng *et al* 1991; Sun *et al* 1994). All these pathways mediate CREB-induced transcription by phosphorylating CREB at residue serine 133 (Ser133) (Gonzalez *et al* 1989). Mutation of this Ser133 residue abolishes the transcriptional response to cAMP *in vitro* (Gonzalez *et al* 1989). The phosphorylated Ser133 binds another protein referred to as the CREB-binding protein (CBP) (Chrivia *et al* 1993). Co-expression of CBP *in vitro* increases the stimulus-induced CREB transcription of CRE reporter gene constructs (Kwok *et al* 1994). CBP is a 265-kDa nuclear protein, which binds to phosphorylated CREB and allows recruitment and stabilization of the RNA polymerase II transcription complex on the promoter of CREB target genes (Nakajima *et al* 1997; Shikama *et al* 2000). CBP is also able to alter the chromatin structure, making the DNA template more accessible to the transcriptional machinery due to its histone acetyltransferase (HAT) activity and ability to recruit other proteins with similar functions (Bannister & Kouzarides 1996; Ogrysko *et al* 1996).

1.13.2 AD4 (SF-1)

The Ad4 site (CCAAGGTC) is also found to be important in the regulation of the bovine *CYP11B* gene. This Ad4 site or homologous sequences have been identified in the regulatory regions of all other steroid P450 genes (*CYP11A1*, *CYP21*, *CYP17*, *CYP11B1*, *CYP11B2*, *CYP19*) (Morohashi *et al* 1992), suggesting an important functional role in steroidogenesis. An AD4-binding protein (AD4BP) has been identified and cloned from bovine adrenal cortex nuclear extract (Morohashi *et al* 1992). AD4BP is a homolog of the steroidogenic factor 1 (SF-1) identified in the

mouse (Ikeda *et al* 1993; Lala *et al* 1992). SF-1 is an orphan member, with an unknown or possibly non-existent physiological ligand, of the nuclear hormone receptor superfamily, with potential phosphorylation sites for cAMP-dependent kinases, CaMK or PKC, suggesting a role for SF-1 in cAMP-dependent transcription. SF-1 is a 53kDa protein comsisting of a zinc finger domain and ligand binding/dimerization domain. These functional domains are conserved between species (human, bovine, rat and mouse) supporting the existence of a ligand. The oxysterols (25-, 26- or 27-hydroxycholesterol) enhance SF-1-dependent transcriptional activity *in vitro*, suggesting that SF-1 is a ligand-activated receptor (Christenson *et al* 1998; Lala *et al* 1997). However, this is a controversial finding (Mellon & Bair 1998) and needs further clarification.

SF-1 is expressed exclusively in steroidogenic tissues and the brain. Important information about the role of SF-1 came from the development of the SF-1 knock-out mouse (Luo *et al* 1994). These mice survive the in utero stage of development but all die within 8 days of birth due to adrenocortical insufficiency. They lack adrenal glands or gonads. Therefore SF-1 plays an essential role in the development and function of the primary steroidogenic tissues. Naturally occuring SF-1 mutants have also been identified in patients with primary adrenal failure (Ozisik *et al* 2002). In males, these mutations are also characterized by complete sex reversal. Within the adrenal, SF-1 has been found to play a key role in the transcriptional regulation of most of the steroid hydroxylase genes (*CYP11A1, CYP21, CYP11B1, CYP17 and CYP19*) as well as *3βHSD* and *StAR*. SF-1 regulation of transcription is mediated by interaction with various co-activator proteins, including steroid receptor coactivator I (SRCI) (Ito *et al* 1998), glucocorticoid receptor interacting protein (GRIPI) (Hammer *et al* 1999) and CBP/p300 (Monte *et al* 1998) and also through repressors such as

DAX1 that inhibits SF-1-mediated steroidogenesis (Babu *et al* 2002; Ito *et al* 1997). SF-1 is homologous to the Drosophila fushi tarazu factor 1 (FTZ-F1) (Lala *et al* 1992), indicating conservation of the gene between vertebrates and invertebrates, again emphasizing the importance of its role in development.

1.13.3 Transcriptional Regulation of CYP11B2

Determination of the important cis-elements within the upstream regulatory region of *CYP11B2* (2017bp) has been carried out using transient transfection of reporter gene constructs in both mouse (Y1) and human (H295R) adrenocortical tumour cell lines. These *CYP11B2*-reporter gene studies confirm that Ang II, K^+, Ca^{2+} channel agonists and cAMP analogues upregulate gene expression (Clyne *et al* 1996; Clyne *et al* 1997). Deletion analysis of this construct has revealed that 3 functionally important cis elements are required for basal expression and response to Ca^{2+} and cAMP. These are a near consensus CRE at -71/-64 (Ad1) and an element at -129/-114 (Ad5) which are homologous to sites previously identified in the bovine *CYP11B* gene, Ad1 and Ad5 respectively, and also a NBRE-1 site at -766/-759 (Bassett *et al* 2004b; Clyne *et al* 1997) (see **figure 1.15**).

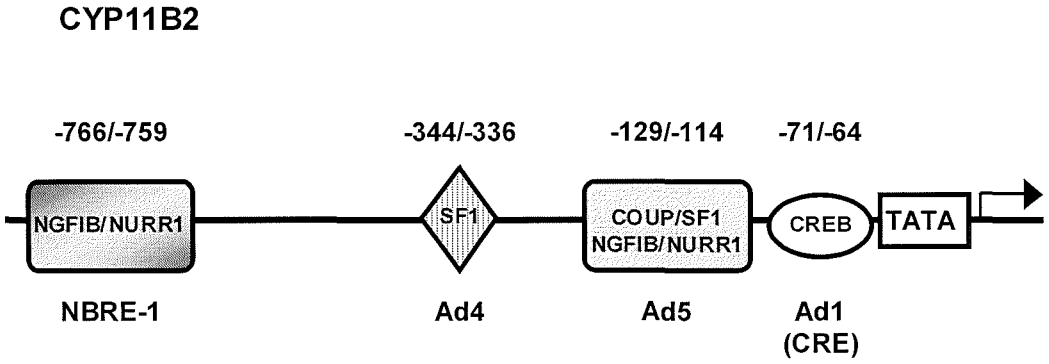


Figure 1.15. Schematic diagram of the transcription factor binding sites in the h*CYP11B2* 5'UTR *(Bassett et al 2004b).*

The CRE at -71/-64 (TGACGTGA) is common to both human (h)*CYP11B2* and (h)*CYP11B1*, and is also present in other species such as the rat, mouse and hamster as well as the bovine *CYP11B* gene (Rainey 1999). The CRE can be regulated by both PKA and CaMK mechanisms (Bassett *et al* 2000; Condon *et al* 2002). Studies of *CYP11B2* CRE binding by electrophoretic mobility shift assay (EMSA) revealed that it binds members of the CREB and ATF (ATF1 and ATF2) families (Bassett *et al* 2000). As mentioned previously, these proteins bind to the CRE following activation by phosphorylation. One possibility is that this phosphorylation is mediated, at least partly, by CaMK I or IV that are induced by Ang II or K+ stimulation (Bassett *et al* 2004b). CaMK I and IV have been shown to phosphorylate CREB and ATF-1, increasing the expression of target genes (Matthews *et al* 1994; Ueda *et al* 1999). Mutations of the CRE site decreased basal reporter gene activity by approximately 50% as well as dramatically reducing the response to Ca^{2+} and cAMP stimulation (Clyne *et al* 1997). However, further deletion studies produced further decreases in transcription suggesting that further sites are neccessary to account for maximal *CYP11B2* expression.

Electrophoretic mobility shift assay (EMSA) analysis of the -129/-114 (Ad5) element (CTCCAGCCTTGACCTT) has shown that it binds several nuclear proteins, including SF-1 and another orphan nuclear receptor, chicken ovalbumin upstream promoter transcription factor (COUP-TF) (Clyne *et al* 1997). On the bovine *CYP17* and the mouse *CYP21* gene, COUP-TF and SF-1 bind competitively to a common site (Lund & Bakke 1995; Rice *et al* 1991). It is not clear at present whether they act competitively or synergistically on the human *CYP11B2* gene. Deletion of this site decreases basal activity by approximately 80% and also reduces the maximal response to Ca^{2+} and cAMP stimulation (Clyne *et al* 1997).

The transcription factors NGFIB (nerve growth factor-induced clone B) and NURR1 (NUR-related factor 1) are members of the NGFIB family of orphan nuclear receptors that bind to a consensus sequence NBRE (AAAGGTCA) (Giguere 1999) (Davis *et al* 1991; Wilson *et al* 1991). These factors can also bind to the Ad5 element (on the non-coding strand) as well as a novel NBRE-1 site at -766/-759 (Bassett *et al* 2004a). Both these transcription factors increase *CYP11B2* expression when co-tansfected in human adrenal H295R cells (Bassett *et al* 2004a). They are both expressed in the zona glomerulosa and their mRNA levels can be upregulated by Ang II or K^+ stimulation. There is also evidence of upregulated expression of NURR1 mRNA that correlates very well with that of *CYP11B2* in subjects with aldosterone producing adenomas (Lu *et al* 2004). Mutation of the NBRE-1 site decreases basal expression as well as the response to Ang II and K^+ (Bassett *et al* 2004a). There are subtle differences in the function of NGFIB and NURR1 and there is evidence to suggest that NURR1 plays a more specific role in the regulation of *CYP11B2* (Bassett *et al* 2004a). Therefore the CRE, Ad5 and NBRE-1 sites interact to regulate basal transcription as well as the response to each signalling pathway (cAMP or Ca^{2+}).

The Ad4 or SF-1 site identified in the bovine gene has now been identified in all the steroid P450s based on sequence alignments, including at position -351/-343 (AGGTCC) of *CYP11B2*. Although this site binds strongly to SF-1, deletion studies suggest it is not essential for basal or stimulus-induced expression of *CYP11B2* (Bassett *et al* 2002). In fact co-expression of *CYP11B2* reporter gene constructs with SF-1 has a negative effect on gene transcription, making the regulation of *CYP11B2* different to the other steroidogenic genes, *StAR*, CYP11A, *CYP11B1* and *CYP17* that are all induced by SF-1. The effects of both NGFIB and NURR1 on *CYP11B2*

expression can be inhibited by SF-1 supporting a negative effect of SF-1 (Bassett *et al* 2004a).

1.13.4 Transcriptional Regulation of CYP11B1

Reporter gene studies and deletion analysis of the regulatory regions of *CYP11B1* showed that two cis-elements (Ad1 and Ad4) are required for basal and ACTH/cAMP induction of *CYP11B1* transcription (Wang *et al* 2000). The Ad1 element at position -71/-64 (TGACGTGA) is a near-consensus CRE also required for the regulation of *CYP11B2* expression. In contrast to *CYP11B2*, the expression of *CYP11B1* is also dependant on the Ad4 or SF-1 binding site (Wang *et al* 2000) (see **figure 1.16**).

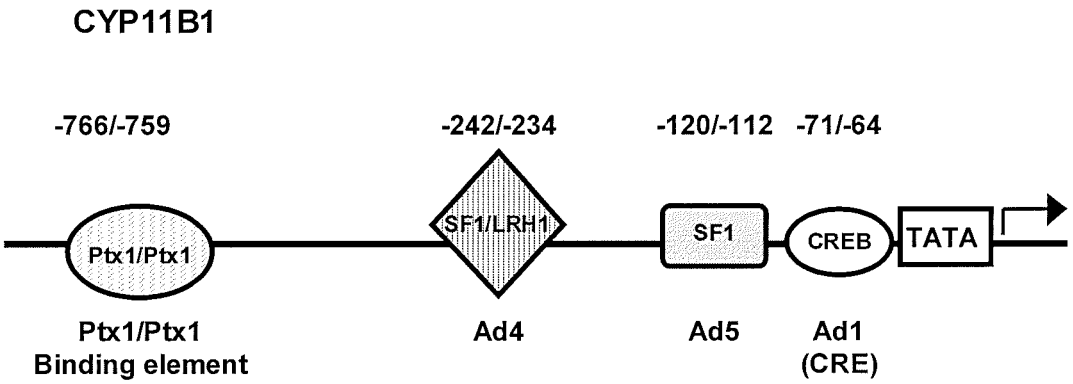


Figure 1.16. Schematic diagram of the transcription factor binding sites in the h*CYP11B2* 5'UTR *(Wang et al 2000; Wang et al 2001).*

Liver receptor homologue-1 (LRH-1) is another member of the orphan nuclear receptor superfamily that has been shown to stimulate *CYP11B1* reporter gene expression (Wang *et al* 2001). LRH-1 is closely related to SF-1 and produces a similar stimulatory effect on *CYP11B1* expression. LRH-1 is mainly expressed in

the liver, intestine, colon and pancreas (Nitta et al 1999) but low levels have been identified in steroidogenic tissues including adrenal tissue as well as in H295R cells (Sirianni *et al* 2002; Wang *et al* 2001). Further analysis revealed that it exerts its positive regulatory effect on *CYP11B1* by binding to the same cis-element as SF-1 (Ad4). Whether LRH-1 acts independently or in parallel with SF-1 is yet to be determined.

A similar effect has been observed with the homeodomain-containing transcription factor pituitary homeobox 1 (Ptx1/Ptx1) (Hiroi *et al* 2003). This factor interacts with SF-1 to mediate pituitary development. Ptx1/Ptx1 has recently been found to be expressed within the adrenal gland and to stimulate *CYP11B1* expression to a similar level as SF-1 after transfection in Y1 cells. Co-expression with SF-1 does not produce a synergistic effect, suggesting independent mechanisms (Hiroi *et al* 2003). The effect of Ptx1/Ptx1 is mediated through the h*CYP11B1* Ptx1/Ptx1 binding element at position -377/372. Mutation of this element decreases the response to Ptx1/Ptx1 but has no effect on basal levels of expression.

These studies highlight specific cis-elements that could account for the different transcriptional regulation of *CYP11B1* and *CYP11B2*. Neither the Ad5 or NBRE-1 elements required for *CYP11B2* regulation are present in *CYP11B1* whereas the -377/372 Ptx1/Ptx1 site appears to be specific to *CYP11B1*. Also SF-1, which is important for basal and stimulated levels of *CYP11B1* expression, actually has a negative effect on *CYP11B2* expression suggesting a possible mechanism for the zonal expression of these isozymes within the adrenal cortex.

As well as being homologous at the nucleotide level, aldosterone synthase ($P450_{aldo}$) and 11β-hydroxylase ($P450_{11\beta}$) are also homologous at the protein level, with only a few amino acids accounting for their differences in structure. The following sections will describe some of the features of cytochrome P450 enzymes highlighting some of the similarities and differences between $P450_{aldo}$ and $P450_{11\beta}$.

1.14 Cytochrome P450 Protein Structure

The $P450_{aldo}$ and $P450_{11\beta}$ proteins both consist of 503 amino acids, including a 24-residue N-terminal mitochondrial targeting sequence. Their apparent molecular masses are 48.5 and 50 kDa respectively (Ogishima *et al* 1991). At the protein level, the two enzymes are 93% homologous with only 37 amino acids that differ between them (Kawamoto *et al* 1992; Mornet *et al* 1989) to account for their different functions. Although the precise 3-dimensional structures of mammalian $P450_{aldo}$ and $P450_{11\beta}$ are yet to be determined, some information can be deduced from the known structures of other cytochrome P450 enzymes.

1.14.1 Bacterial/Microsomal P450 homologues

To date, no structure of a mitochondrial cytochrome P450 has been experimentally determined. The tertiary structures of several bacterial P450s have been well studied, including P450cam (CYP101), P450BM3 (CYP102) and P450terp (CYP108). More recently the structures of three mammalian microsomal P450s, CYP2C5 (Williams *et al* 2000), CYP2C8 (Schoch *et al* 2004) and CYP2C9 (Williams *et al* 2003) were also resolved. Studies of bacterial homologues suggest that P-450 enzymes consist of a number of helices (A-L) (Ravichandran *et al* 1993). The haem in cytochrome P-450 enzymes is located between helices I and L.

1.14.2 Regions of Conservation

Although the sequence homology among the P450s is limited to 10-30% (Hasemann *et al* 1995) (Denisov *et al* 2005), they all appear to have a similar tertiary structure with a conserved structural core (Peterson & Graham 1998). This comprises a four-helix bundle of helices D, E, I and L, as well as regions of helices J and K and the structurally conserved β-sheets 1 and 2. CYP450 enzymes contain haem prosthetic groups and use iron-oxo intermediates for oxidative reactions (see **section 1.7**). The inner helices form a 'pocket' around the haem, enabling substrate binding and activation. This haem-binding domain is a region of conserved non-polar residues that establish a hydrophobic environment surrounding the haem that is important in maintaining its full redox potential. It consists of the B'-C loop, the central I helix, the L-helix and the cys pocket (Hasemann *et al* 1995). The cys pocket is the β bulge region that surrounds the cysteine residue that is absolutely conserved in all P450 enzymes. This conserved cysteine residue forms the proximal or fifth ligand to the haem iron and is responsible for the characteristic name; the ferrous-CO complex gives 450nm Soret absorbance (Denisov *et al* 2005). The structurally conserved sets of β sheets form the hydrophobic substrate access channel. All of the P450 structures also have highly conserved coil, termed the meander, of approximately 20 amino acids that runs between the K' helix and the cys pocket. It interacts with the ERR triad (Glu(439)-Arg(442)-Arg/His/Asp(498)) located within the K helix forming a set of salt bridges that are thought to be important in stabilizing the core structure. Disruption of this motif results in a loss of haem binding (Hasemann *et al* 1995).

1.14.3 Regions of Variability

The regions of the highest sequence variability among the CYP450s are thought to be important for substrate recognition or redox partner association. These variable regions tend to form the N terminal part of the protein and include helices A, B, B', F and G and their interconnecting loops. Gotoh determined several regions thought to be important for substrate binding through analysis of the known structure of P450cam and sequence alignment with members of the mammalian CYP2 family. He called these regions substrate recognition sites (SRS) 1-6 (Gotoh 1992).

SRS	Secondary structure	Residues
SRS-1	B' helix and B'-C loop	103-106
SRS-2	C terminus of helix F	209-216
SRS-3	N terminus of helix G	248-255
SRS-4	N terminus of helix I	302-320
SRS-5	β sheet 3 (β3)	375-385
SRS-6	β sheet 5 (β5)	485-493

The positioning of these SRSs is supported by the identification of residues essential for CYP2 substrate recognition that map to the identified sites (Gotoh 1992).

1.14.4 Homology Modelling

Homology modelling of aldosterone synthase ($P450_{aldo}$) and 11β-hydroxylase ($P450_{11\beta}$) using the known structures of the bacterial enzymes P450BY-3 (*CYP102*)

and P450terp (*CYP108*) produced models with a very similar overall structure but with differences in the position of the haem (see **figure 1.17**). The positions of the various α-helices and β-sheets within these models are shown in **table 1.2**. (Belkina *et al* 2001).

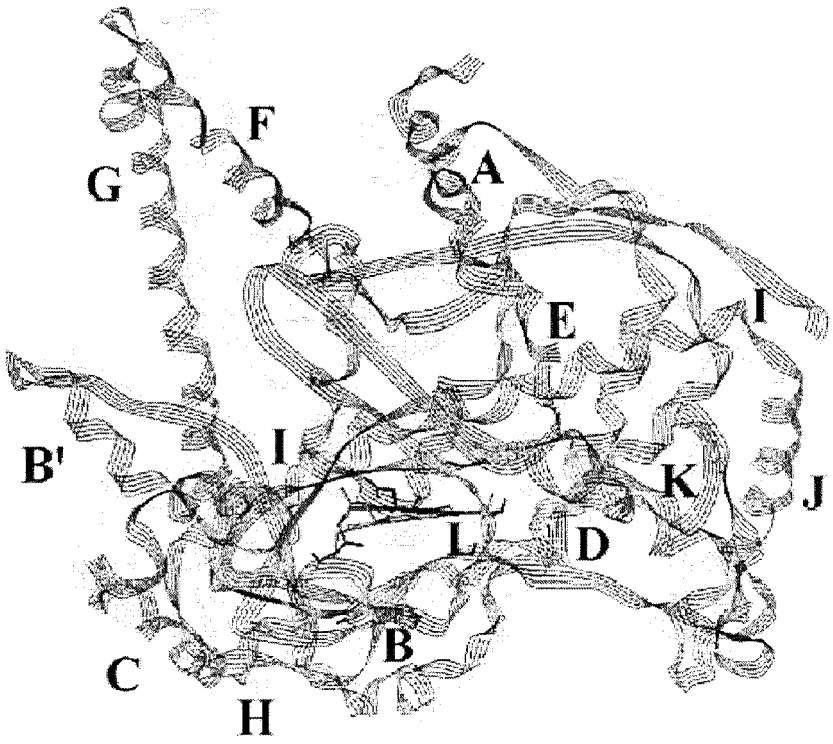


Figure 1.17. Superposition of the homology models of $P450_{11\beta}$ (black) and $P450_{aldo}$ (grey). The positions of each of the α-helices are marked. While the overall structure is similar the position of the haem differs between the two proteins. *(Taken from Lisurek & Bernhardt 2004)*

Name	$P450_{11\beta}$	$P450_{aldo}$	Name	$P450_{11\beta}$	$P450_{aldo}$
A-helix	30-44	30-40	F-helix	223-237	224-243
A1-helix	46-50	46-50	G-helix	247-282	249-281
β1-1	55-59	55-57	H-helix	289-296	290-296
β1-2	64-67	65-67	I-helix	301-331	301-331
B-helix	70-75	71-76	J-helix	334-354	334-351
B'-helix	94-102	94-102	K-helix	364-376	365-376
B3-helix	115-118	115-118	β1-3	401-405	401-408
C-helix	127-140	127-140	β1-4	383-386	380-386
C'-helix	145-150	145-150	β2-1	389-392	389-393
D-helix	158-175	157-175	β2-2	395-398	395-398
E'-helix	185-192	185-192	K'-helix	406-410	406-416
E-helix	196-206	195-204	L-helix	450-468	451-466
D3-helix	209-214	207-213			

Table 1.2. Secondary structures of the $P450_{11\beta}$ and $P450_{aldo}$ 3D homology models *(Belkina et al 2001)*

Following targeting to the mitochondrion, $P450_{aldo}$ and $P450_{11\beta}$ bind to the inner membrane through interactions that are not yet well defined. However, from the homology models, there appear to be hydrophobic patches on the protein surfaces, that encompass helices A and A'. These are thought to be involved in protein-membrane interactions.

The I helix, the largest helix running through the centre of the molecule, contains many hydrophobic amino acids and forms the major part of the putative active site, essential for substrate binding and specificity (Belkina *et al* 2001). Excluding the mitochondrial targeting peptide, only 29 amino acids differ between aldosterone synthase and 11β-hydroxylase out of a total of 479. Several of these distinguishing amino acids are located within the I, helix including amino acids at positions 301, 302 and 320. Replacing these amino acids within $P450_{aldo}$ with the corresponding $P450_{11\beta}$ equivalents (L301P, E302D, A320V) individually, enhances the 11β-hydroxylation activity of $P450_{aldo}$ by approximately 2 fold. When all 3 mutations are

present as a triple mutant, there is a synergistic effect with an increase in 11β-hydroxylase activity to 85% of wildtype $P450_{11\beta}$ and a suppression of aldosterone synthase activity to 10% of wildtype $P450_{aldo}$ (Bottner & Bernhardt 1996). In the opposite scenario, exchanging amino acids within $P450_{11\beta}$ for the $P450_{aldo}$ equivalents to produce the mutants Val320Ala, Ser288Gly and Asn335Asp (either alone or in combination) confers aldosterone synthase activity to $P450_{11\beta}$ with no apparent affect on its 11β-hydroxylase function (Bottner & Bernhardt 1996; Curnow *et al* 1997; Mulatero *et al* 1998). Therefore, the differences in amino acid sequence within the I helix may largely explain the differences in specificity of the two enzymes.

The I helix within $P450_{aldo}$ or $P450_{11\beta}$ is encoded by residues from exon 5 and exon 6 of the genes. Studies on the monogenic hypertensive disorder, glucocorticoid suppressible hyperaldosteronism (GSH) support an essential role of this protein region for enzyme function (see **section 1.5.2.1**). GSH is the result of a chimeric gene consisting of the 5' region of *CYP11B1* attached to part of *CYP11B2*, resulting in the synthesis of aldosterone synthase being under the control of ACTH. However, if the cross-over point between the genes is after codon 302 (half way through exon 5), aldosterone synthase activity is undetectable, suggesting that regions upstream of this are indeed essential for enzyme function (Pascoe *et al* 1992a).

Homology modelling has suggested that the main difference between $P450_{aldo}$ and $P450_{11\beta}$ is the orientation of the haem. This affects the way in which the haem interacts with the side chains of adjacent amino acids. In $P450_{11\beta}$, the haem interacts with Arg448 and Arg384 whereas in $P450_{aldo}$, the haem forms two interactions with Arg448. Mutations in both Arg448 and Arg384 have been identified as the cause of

congenital adrenal hyperplasia (11β-hydroxylase deficiency) (see **section 1.15.2.3**) with all known mutations completely abolishing enzyme activity (Curnow *et al* 1993; Geley *et al* 1996; Nakagawa *et al* 1995; White *et al* 1991), supporting a key role for these amino acids in haem stabilization. Due to these differences in haem interactions, the active site of $P450_{11\beta}$ is predicted to be larger than that of $P450_{aldo}$ (Belkina *et al* 2001). This would seem appropriate as the main substrate for $P450_{11\beta}$ (S) is larger than that for $P450_{aldo}$ due to the presence of an extra 17α-hydroxy group.

Other studies clearly demonstrate that residues relatively remote from the known active site of $P450_{aldo}$ or $P450_{11\beta}$ can also markedly alter enzyme function. Their location provides further insights into the function of cytochrome P450 enzymes. Fisher et al (2000) showed that substituting the aldosterone-specific residue D147, that is distant from the active centres, for the 11β-hydroxylase equivalent (E147) and vice versa, significantly alters activity (Fisher *et al* 2000).

1.15 Evidence for a Role of the *CYP11B* locus in Essential Hypertension.

As early as 1956, functional abnormalities of the adrenal cortex were suggested as a cause of essential hypertension (Genest *et al* 1956) and adrenal cortex hyperplasia has been found to be a feature of many hypertensive patients at post-mortem (Komiya *et al* 1991). Although no single causative defect in corticosteroid biosynthesis has been identified, evidence from animal models and monogenic forms

of hypertension demonstrate that modification of the *CYP11B1* and *CYP11B2* genes can affect blood pressure and cardiovascular function and may therefore help to identify possible mechanisms contributing to essential hypertension.

1.15.1 Animal Models of Hypertension

Animal models have proved extremely useful in studying the heritable component of essential hypertension. The complexity of interpreting data from human subjects is greatly reduced by using inbred strains, which decreases the genetic heterogeneity, and also environmental influences can be completely controlled. Several rat models of genetic hypertension have been developed (Rapp 2000). Genetic crosses between hypertensive and control strains enable the identificaton of the individual genetic causes of hypertension.

1.15.1.1 Dahl salt sensitive rat

In 1962, Dahl selectively bred rats according to the level of blood pressure achieved on a high salt (NaCl) diet. Two strains of rat were selected. In one strain, blood pressure is salt sensitive (ss) and rats become severely hypertensive upon salt loading. In the other, blood pressure is salt resistant (sr). Biochemical analysis revealed differences in corticosteroid metabolism. In the rat, the principal glucocorticoid is corticosterone, which is synthesised from DOC by 11β-hydroxylase. 11β-hydroxylase is also able to catalyze 18-hydroxylation and an alternative end product is 18-hydroxydeoxycorticosterone (18-OH DOC), which may have weak mineralocorticoid activity. The ratio of corticosterone to 18-OH-DOC varies and may be due to variations in 11β-hydroxylase structure and function. In the ss strain adrenal cortex, there is a proportionally greater synthesis of 18-OH DOC

relative to corticosterone (Rapp & Dahl 1972). Investigating the *CYP11B1* gene, which encodes for 11β-hydroxylase as a possible candidate gene, revealed polymorphisms which associate with 18-OH DOC levels as well as with blood pressure. The 11β-hydroxylase in the sr strain has 5 mutations in exons 2, 6, 7 and 8 (Cicila *et al* 1993). A combination of two of these, V381L and I384L, causes a loss of 11 β-hydroxylase function *in vitro* and is thought to be the explanation of why the sr animals synthesise less 18-OH-DOC (Matsukawa *et al* 1993; Nonaka *et al* 1998). A chromosome 7 congenic strain of Dahl rats confirms that this region contains a QTL for blood pressure, with the *CYP11B1* gene being the likely candidate (Cicila *et al* 1997). Mutations have also been identified in *CYP11B2* of the Dahl rat (Cover *et al* 1995). However, these appear to cause a decrease in aldosterone synthase activity in the ss animal and so their contribution to the hypertensive phenotype is unclear. The Dahl rat is an excellent example of how genetic differences, in this case resulting in altered corticosteroid production, can lead to hypertension. It is also a good model of how environmental factors (salt) and genetic factors can interact to produce a hypertensive phenotype.

1.15.2 Mendelian Hypertensive Conditions

Several genes have been identified that cause Mendelian forms of hypertension in man. These disorders are caused by single gene defects that result in severe, early onset hypertension. Although rare, these mutations, which all result in altered renal salt reabsorption, give insight into possible molecular pathways involved in the pathogenesis of hypertension (Lifton *et al* 2001). Of particular interest to this review are those involving variation at the *CYP11B* locus.

1.15.2.1 Glucocorticoid suppressible hyperaldosteronism (GSH)

Glucocorticoid suppressible hyperaldosteronism (GSH) is an autosomal dominant condition characterized by elevated aldosterone levels despite suppressed plasma renin together with hypertension and hypokalaemia (Rich *et al* 1992; Sutherland *et al* 1966). The aldosterone levels follow a circadian rhythm identical to that of ACTH and can be treated successfully with a synthetic glucocorticoid (dexamethasone) that inhibits ACTH production from the pituitary gland. GSH is caused by a chimeric gene that forms when the 11β-hydroxylase (*CYP11B1*) and the aldosterone synthase (*CYP11B2*) genes become misaligned during meiosis and crossing over occurs (Lifton *et al* 1992). The chimera has a 5′ end containing the promoter region of the *CYP11B1* gene and exons 1-3 joined to exons 5-9 of *CYP11B2*. This results in the gene expression and enzymatic activity of aldosterone synthase being controlled by ACTH instead of Ang II and which therefore does not respond to Na^+ status. Ectopic secretion of aldosterone from the adrenal fasciculata increases salt and water reabsorption and raises blood pressure. Substitution of more than the first 3 exons of *CYP11B1* results in an inactive chimeric gene suggesting residues after exon 3 are crucial to aldosterone synthase activity.

1.15.2.2 Apparent Mineralocorticoid Excess (AME)

Apparent mineralocorticoid excess (AME) is an autosomal recessive trait characterized by early onset hypertension with hypokalemia and metabolic alkalosis, suppressed renin and very low circulation levels of aldosterone. Despite the low levels of aldosterone, hypertension in these subjects responds to mineralocorticoid receptor blockade. Genetic analysis of affected families revealed an association between the disease and loss of function mutations in the gene for 11β-

hydroxysteroid dehydrogenase type II (11βHSD2) (Mune *et al* 1995). In normal subjects, circulating cortisol levels are up to 1000 fold greater than circulating aldosterone levels. Cortisol has a similar affinity for the MR as aldosterone but is prevented from binding under normal circumstances by the enzyme 11βHSD2 which converts it to the inactive steroid, cortisone (Funder *et al* 1988; Stewart *et al* 1987) (see **section 1.9.3**). When 11βHSD2 is inactivated, cortisol is free to activate MR resulting in hypertension.

1.15.2.3 11β-hydroxylase deficiency

Congenital adrenal hyperplasia is the generic name given to a group of inherited disorders resulting from mutations in the enzymes involved in corticosteroidogenesis (see **section 1.8**). 11β-hydroxylase deficiency is the second most common cause of congenital adrenal hyperplasia, accounting for 5-8% of cases in the general population (White *et al* 1992) with a higher frequency in an Israeli population of Moroccan Jewish origin (Rosler *et al* 1992). It is an autosomal recessive condition resulting from inactivating mutations in the *CYP11B1* gene, which causes adrenal hyperplasia due to the increased drive of ACTH attempting to maintain normal circulating cortisol levels. The result is low-renin hypertension and hypokalemia due to the accumulation of the precursor DOC, which has mineralocorticoid properties. The circulating levels of DOC are normally too low to activate the MR but in this condition result in mineralocorticoid excess. The accumulating precursor steroids are shunted into pathways of androgen biosynthesis, causing female virilization. and postnatal hyperandrogenism in both sexes (White & Speiser 1994).

Mutations causing 11β-hydroxylase deficiency

The disruptive mutations are distributed across the entire gene but with a slightly higher frequency in exons 2, 6, and 8. There also appear to be mutational 'hot spots' in codons 318, 384 and 448 (see **table 1.3**). Several mutations disrupt the reading frame of the coding sequence, resulting in the biosynthesis of a truncated non-functional protein. These include nonsense mutations; W116X, K174X, W247X, Q338X, Q356X or frameshift mutations, delC32, 2bp insertion at codon 394, 28bp deletion or a 5bp insertion in exon 2 (see **table 1.3**). The complete loss of enzymatic activity is usually explained by mutations, which result in the haem-binding or substrate-binding domains being incomplete or absent. Many of the missense mutations in exons 2, 5, 6, 7 and 8 have also been shown to abolish 11β-hydroxylase activity completely after transfection in cell culture; V129M, T318M, A331V, E371G, R374Q, R384Q, V441G and R448C (Curnow *et al* 1993; Geley *et al* 1996).

In vitro analysis of mutations Pro42S, Asn133His and Thr319Met showed that they partially reduced 11β-hydroxylase activity to 15-37% of the wildtype activity (Joehrer *et al* 1997). These mutations were identified in subjects with a non-classic or mild form of 11β-hydroxylase deficiency. It is characterized by milder androgen excess with slightly elevated blood pressure in some cases. The symptoms of advanced bone age, accelerated growth, acne and menstrual cycle abnormalities may develop during childhood or at puberty. A similar non-classic form of 21-hydroxylase deficiency also exists, and in fact occurs more frequently than the classic form (Merke & Bornstein 2005). Therefore it is likely there are more cases of non-classic 11β-hydroxylase deficiency that have not been identified due to the relatively mild signs and symptoms of androgen excess.

Merke et al identified three novel mutations causing 11β-hydroxylase deficiency, a nonsense mutation at codon 19, a novel missense mutation in codon 318 (Thr318Arg) and a G to A substitution at the first base pair of intron 5 (318+1A-G) within the splice site (Merke *et al* 1998). These mutations suggest that splice site variants may be important causative mechanisms of 11β-hydroxylase deficiency and also identify codon 318 as an additional mutational 'hot spot'. Merke et al (1998) also identified two apparent polymorphisms within the *CYP11B1* gene of healthy control subjects. These turned out to be gene conversions events in exon 1 (R43Q) and exon 7 (A386V) whereby the 11β-hydroxylase-specific residue had been replaced with the corresponding aldosterone synthase-specific residue. The frequency of these or similar gene conversion events within the general population is unknown.

Mutation	Exon	Putative 3D location	Effect *in vitro*	Reference
Gln19STOP	1	N terminus	Truncated protein	Merke et al (1998)
delC32	1	A-helix	Complete loss of activity	Curnow et al (1993)
Pro42Ser	1	A-helix	15% ↓ activity	Joehrer et al (1997)
del28bp (S105-L113)	2	B'-B3 loop	Truncated protein	Skinner et al (1996)
Trp116Cys	2	B-C loop	97% ↓ activity	Krone et al (2005)
Trp116STOP	2	B-C loop	Complete loss of activity	Naiki et al (1993)
Ins 5bp 121	2	C-helix	Truncated protein	Skinner et al (1994)
Val129Met	2	C-helix	Complete loss of activity	Geley et al (1996)
Asn133His	3	C-helix	17% ↓ activity	Joehrer et al (1997)
Lys174STOP	3	D-helix	Truncated protein	Curnow et al (1993)
Trp247STOP	4	G-helix	Truncated protein	Geley et al (1996)
Gly267Arg	5	G-helix	May alter splicing	Skinner et al (1996)
Leu299Pro	5	Start of I helix	99% ↓ activity	Krone et al (2005)
Thr318Met	5	I-helix	Complete loss of activity	Curnow et al (1993)
Thr318Arg	5	I-helix	Undetermined	Merke et al (1998)
318+1G-A	5+1	-	Altered splicing	Merke et al (1998)
Thr319Met	6	I-helix	37% ↓ activity	Joehrer et al (1997)
Ala331Val	6	I-helix	Complete loss of activity	Geley et al (1996)
Gln338STOP	6	J-helix	Truncated Protein	Curnow et al (1993)
Val348Ala	6	J-helix	Undetermined	Skinner et al (1994)
Gln356STOP	6	BetweenJ-K helix	Truncated Protein	Curnow et al (1993)
Glu371Gly	6	K-helix	Complete loss of activity	Geley et al (1996)
Arg374Gln	6	K-helix	Complete loss of activity	Curnow et al (1993)
Arg384Gln	7	Haem side chain	Complete loss of activity	Curnow et al (1993)
Arg384Gly	7	Haem side chain	Undetermined	Nakagawa et al (1995)
Ins2bp394	7	β1-4	Truncated Protein	Helmberg et al (1992)
Arg427His	8	Meander	May affect haem binding	Skinner et al (1994)
delF438	8	Meander	Complete loss of activity	Krone et al (2005)
Val441Glu	8	Meander	Complete loss of activity	Curnow et al (1993)
Arg448Cys	8	Haem side chain	Complete loss of activity	Geley et al (1996)
Arg448His	8	Haem side chain	Complete loss of activity	White et al (1991)
Ins3bp464	8	L-helix	Complete loss of activity	Geley et al (1996)

Table 1.3. Mutations in *CYP11B1* causing 11β-hydroxylase deficiency

The reason for the disruptive effects of many of these missense mutations can be hypothesised from their putative 3D locations. As mentioned previously, in $P450_{11\beta}$ the haem interacts with Arg448 and Arg384 and mutations at these sites completely abolish enzyme activity (Curnow *et al* 1993; Geley *et al* 1996; Nakagawa *et al* 1995; White *et al* 1991). The residue T318 lies within the I-helix or putative active site and is completely conserved in all CYP450s. It is thought to be essential for binding of molecular oxygen and/or proton transfer, which are essential for enzyme activity (Curnow *et al* 1993; Merke *et al* 1998). Amino acid residues E371 and R374 within the K helix form part of the ERR-triad and so mutations at these sites would be expected to cause enzyme destabilization (Hasemann *et al* 1995). Residue P42 within the A helix is conserved in all *CYP11B* isoenzymes and thought to be essential for proper orientation of the enzyme in the mitochondrial membrane (Joehrer *et al* 1997). Amino acid N133, also conserved in the *CYP11B* family, lies within a region that may form part of the substrate access channel (Joehrer *et al* 1997). Within this region, there is also a cluster of glycines (G123, G128, G134), which are conserved between $P459_{11\beta}$ and $P450_{aldo,}$ and are involved in the flexibility of the B-C loop. The B-C loop is thought to be important for substrate recognition as it contains the proposed SRS-1 (Gotoh 1992) (see **section 1.14**). Mutations within this region, such as W116, would be expected to affect substrate binding (Krone *et al* 2005). Residue L229 is located very close to the I-helix/active site. The introduction of a proline, which is a large cyclic residue, at this site may alter the position of the helix relative to the haem, disrupting enzyme function (Krone *et al* 2005). Other residues such as V129 and A331, although their exact role is unknown, they are conserved between the *CYP11B* isoenzymes and so can be indirectly assumed to be functionally important.

Although the frequency of 11β-hydroxylase deficiency within the general population is very rare, it draws attention to the *CYP11B1* locus as a possible candidate that may contribute to hypertension. Also, the more recent identification of mutations causing a milder non-classic 11β-hydroxylase deficiency highlights the possibility of the existence of mutations that cause very subtle differences in enzyme activity that may be more difficult to recognise by phenotypic analysis.

1.15.3 Mendelian Hypotensive Conditions

As well as the hypertensive conditions, there are also several Mendelian forms of hypotension resulting from loss-of-function mutations in 9 different genes that impair renal sodium reabsorption (Lifton 2001). These include the genes encoding aldosterone synthase (Pascoe at al 1992), 21-hydroxylase (Amor et al 1988), the mineralocorticoid receptor (Geller et al 1998) and the subunits of the ENaC (Chang et al 1996).

1.15.3.1 Aldosterone synthase deficiency

Aldosterone synthase deficiency usually presents in newborn infants as a life threatening electrolyte imbalance. It is characterised by salt wasting and hyperkalemia with elevated plasma renin activity and decreased aldosterone levels, resulting in dehydration, vomiting and failure to thrive. There are two types of aldosterone synthase deficiency, previously thought to be two separate enzyme deficiencies and referred to as corticosterone methyl oxidase type I (CMOI) and type II (CMOII) (Ulick 1976). However, the realization that aldosterone synthase is responsible for all 3 of the final stages of aldosterone synthesis (11β-hydroxylation, 18-hydroxylation and 18-oxidation) (see **section 1.8.7**) indicates that both types must

be caused by mutations in the *CYP11B2* (aldosterone synthase) gene. Type I deficiency appears to disrupt 18-hydroxylation as indicated by low to normal secretion of 18-hydroxycorticosterone and little or no aldosterone production. Type II affects 18-oxidation resulting in low or normal aldosterone levels at the expense of increased secretion of its immediate precursor, 18-hydroxycorticosterone (Ulick *et al* 1992b). The largest numbers of Type II cases identified have been Iranian Jews, born from consanguineous parents, but the disease has also been documented throughout Europe and North America.

Mutations causing aldosterone synthase deficiency

Aldosterone synthase deficiency is a rare autosomal recessive condition arising from mutations within the *CYP11B2* gene. Like 11β-hydroxylase deficiency the causative mutations are spread throughout the entire gene, in this case *CYP11B2*, but with a tendency to cluster in exon 3 (see **tables 1.4a and b**). However, the number of mutations reported to cause aldosterone synthase deficiency is less than those reported to cause 11β-hydroxylase deficiency.

TYPE I				
Mutation	**Exon**	**Putative 3D Location**	**Effect *in vitro***	**Reference**
Val35del5bp	1	A-helix	Truncated protein	Mitsuuchi et al (1993)
6bp duplication 153	3	Between C'-D helix	Complete loss of activity	Kayes-Wandover et al (2001)
Glu255STOP	3	G-helix	Truncated protein	Peter et al (1997)
Tyr265STOP	3	G-helix	Truncated protein	Lopez-Siguero et al (1999)
Leu324Gln	6	I-helix	Undetermined	Lopez-Siguero et al (1999)
Arg384Pro	7	β1-4	Complete loss of activity	Geley et al (1995)
Val386Ala/Glu 188Asp	7/3	β1-4/E'-helix	Complete loss of activity	Lopez-Siguero et al (1999)
Leu461Pro	8	L-helix	Complete loss of activity	Nomoto et al (1997)
Arg173Lys/ Glu198Asp/Val 386Ala	7/3/3	β1-4/D-and E-helix	↓11β and 18-hydrox, no 18-oxidase activity	Portrat-Doyen et al (1998)

Table 1.4a. Individual and combined mutations in *CYP11B2* causing Type 1 aldosterone synthase deficiency (CMO-I)

TYPE II				
Mutation	**Exon**	**Putative 3D Location**	**Effect *in vitro***	**Reference**
Val386Ala/Arg 181Trp	7/3	β1-4/between D-andE-helix	0.2% activity of Wt	Pascoe et al (1992)
Thr185Ile	3	E-helix	↓ 18OH No 18Ox	Peter et al (1998) Lisurek et al (2004)
Arg181Trp/del C372	3/	BetweenD-E and K-helix	Complete loss of activity	Zhang et al (1995)
Val386Ala/Thr3 18Met	7/5	I-helix	Complete loss of activity	Zhang et al (1995)
Exon 3+4 conversion	3/4	-	Normal aldosterone	Fardella et al (1996)
T498A	9	C-terminus	11β and 18-hydrox, no 18-oxidase activity	Dunlop et al (2003)

Table 1.4b. Individual and combined mutations in *CYP11B2* causing Type 2 aldosterone synthase deficiency (CMO-II)

Most of the mutations causing Type I aldosterone deficiency result in a complete loss of aldosterone synthase activity *in vitro* (see **table 1.4a**). They include the 5bp deletion in exon 1 that results in a premature stop codon (Mitsuuchi *et al* 1993), the nonsense mutation in exon 4 (E255X) which produces an enzyme lacking the haem binding domain (Peter *et al* 1997), and a missense mutation in exon 8 (L461P) also thought to be important for haem-binding (Nomoto *et al* 1997). The substitution R384P also causes Type I deficiency (Geley *et al* 1995). This arginine is conserved in all mitochondrial P450 enzymes and is thought to interact with the haem group (see previous **section 1.14.2**). A similar mutation has been identified in *CYP11B1* that also completely abolishes activity (Curnow *et al* 1993).

Many of the mutations causing type II deficiency, result in mildly impaired activity or only affect certain functions of aldosterone synthase (see **table 1.4b**). For example, Thr185Ile increases 11β-hydroxylase activity while severely reducing 18-hydroxylation and abolishing aldosterone synthase activity (Lisurek & Bernhardt 2004). Interestingly, *CYP11B2* and *CYP11B1* are identical at this position. The substitution T498A retains 11β-hydroxylase and 18-hydroxylase activities but not 18-oxidase activity, coinciding with the clinical diagnosis of type II deficiency (Dunlop *et al* 2003). These mutations give insights into which residues are important for the different functions of aldosterone synthase.

However, there is some inconsistency between the *CYP11B2* activity as assessed *in vitro* and the clinical phenotype observed (Portrat-Doyen *et al* 1998). For example, the mutations reported by Zhang et al were present in a compound heterozygote with Arg181Trp/delC372 on one allele and Val386Ala/Thr318Met on the other. The clinical symptoms were suggestive of Type II aldosterone deficiency, with detectable

serum aldosterone and elevated 18-hydroxycorticosterone, but the mutants had no measurable aldosterone synthase activity *in vitro* (Zhang *et al* 1995). This may be explained by other *in vitro* studies demonstrating higher aldosterone production using a *CYP11B1*-containing vector compared to empty-vector, implying that *CYP11B1* may be capable of very low levels of aldosterone synthesis (Dunlop *et al* 2003). However, the opposite situation occurs. The presence of mutations R173K/E198D/A386V produces a phenotype consistent with Type I aldosterone synthase deficiency but *in vitro* studies showed residual activity comparable to Type II deficiency (Portrat-Doyen *et al* 1998). Hence, the complications and dispute over the characterization of the two subtypes of aldosterone synthase deficiency.

In several cases, the simultaneous presence of more than one homozygous mutation is required to produce the phenotype of aldosterone synthase deficiency. Individually, several mutants such as R173K, V386A, E198D or R181W have only modest effects on enzyme activity *in vitro* but produce much greater effects when present in combination, suggesting there is significant interaction between the mutant loci (Pascoe *et al* 1992b; Portrat-Doyen *et al* 1998; Zhang *et al* 1995). Indeed, R173K and V386M occur frequently in most populations studied, which supports their minor effects on enzyme activity. However, when present, together they must interact to induce a conformational change in the enzyme, resulting in altered activity. In some subjects with apparent clinical symptoms of aldosterone synthase deficiency, no mutation affecting P450aldo activity could be identified (White 2004). The causative gene(s) in these cases remains to be determined.

The putative 3D locations of several of these mutants are within the conserved P450 structural core, such as residues 173 (helix D), 318 (helix I), 324 (helix I), 386

(strand 4 of β-sheet 1) and 461 (helix L), confirming an essential functional role of these regions of the enzyme. However, although these mutations give key insights into what residues are essential for enzyme function, they do not give enough information to explain fully the relationship between gene/protein structure and enzyme activity.

All these Mendelian disorders are very rare and do not explain the overall blood pressure variation in the general population. However, they do provide an insight into the various pathways through which genetic variation can alter blood pressure.

In the light of these rare syndromes it is of interest that a slightly impaired 11β-hydroxylase function has been previously found in subjects with essential hypertension compared to controls. A study 20 years ago by de Simone and colleagues reported an increase in the levels of the precursor DOC following ACTH stimulation in hypertensive patients compared to controls (de Simone *et al* 1985). More recently, a similar observation was made in hypertensives from Italy who had an elevated ratio of 11-deoxycortisol to cortisol, which is a marker of 11β-hydroxylase activity (Connell *et al* 1996). **Section 1.10.2.1** described how dysregulated aldosterone synthesis, as determined by an elevated ratio of aldosterone to its principle trophin renin, appears to be a common feature of essential hypertension. Subsequent to the preceding discussion of widespread variation in *CYP11B1* and *CYP11B2*, what is the possibility that similar genetic variant(s) contribute to the predisposition to essential hypertension? This is the subject of the final section.

1.16 Possible Genetic Basis for Hypertension with an Elevated ARR

As mentioned previously there is now evidence that altered aldosterone production resulting in an elevated ARR may be a common feature of essential hypertension (Lim *et al* 2002a). Hypertension with a raised ARR may be a subset of hypertension that develops gradually as a result of the interaction between environmental and genetic influences (Connell *et al* 2003). The potential role for corticosteroids in the development of hypertension has previously been discussed. The enzymes aldosterone synthase (encoded by *CYP11B2*) and 11β-hydroxylase (encoded by *CYP11B1*) are responsible for the last stages in the synthesis of aldosterone and cortisol respectively. The monogenic forms of hypertension (**section 1.15.2**) and animal models such as the Dahl rat (**section 1.15.1**), mentioned previously, highlight both these genes as key candidates for hypertension.

1.16.1 CYP11B2 Polymorphisms and Hypertension

Two common variants have been described in the *CYP11B2* gene (White & Slutsker 1995). The first is a single nucleotide (cytosine/thymidine) substitution in the 5′ promoter region at position -344C/T that disrupts a putative binding site for the steroidogenic factor SF-1. The other involves the second intron of *CYP11B2*, part of which is converted to the intron 2 sequence found in the adjacent gene *CYP11B1*.

These variants are in tight linkage disequilibrium so that the common haplotypes are T/Conv (38%), C/Wt (45%) and T/Wt (16%) (Davies *et al* 1999). It has previously been reported that the SF-1 T allele and the intron conversion allele occur more frequently in hypertensive subjects than in normotensives (Brand *et al* 1998; Davies

et al 1999), especially those with a high ARR (Lim *et al* 2002b), although this finding is not supported in all studies (Kupari *et al* 1998; Mulatero *et al* 2000). The association between the SF-1 T allele and blood pressure is supported by evidence of an epistatic interaction of this locus with the Y chromosome on the likelihood of developing higher blood pressure (Charchar *et al* 2002). These polymorphisms also appear to be associated with higher average aldosterone levels, as measured by excretion rates of its principle metabolite tetrahydroaldosterone (Davies *et al* 1999), as well as higher average plasma aldosterone levels (Paillard *et al* 1999). Again, this finding has not always been confirmed (Pojoga *et al* 1998). As mentioned previously, recent studies demonstrate that up to 15% of unselected patients with hypertension have a raised aldosterone to renin ratio suggesting that altered regulation of aldosterone production may be a common factor in essential hypertension (Lim *et al* 1999b). However, *in vitro* studies suggest that, although the T allele at -344 has a 4 fold decreased affinity for SF-1, this has no effect on gene transcription (Bassett *et al* 2002). This strongly suggests that it is not the functional variant and that it may be in linkage disequilibrium with the as yet unidentified functional variant(s). The reduced binding of SF-1 may enable more to bind to a functional site elsewhere.

1.16.2 CYP11B2 and altered 11β-Hydroxylation

Other studies have focused on the relationships of these *CYP11B2* polymorphisms to basal and ACTH-stimulated corticosteroid levels (Davies *et al* 2001). No correlation was found between the genotypes of either polymorphism and basal corticosteroid levels. Similarly, no relationship of genotype was seen to the response of the end products cortisol and corticosterone to ACTH stimulation. However, their respective

precursors, 11-deoxycortisol and deoxycorticosterone, showed a significantly greater response to ACTH stimulation in those subjects with the SF-1 T allele and the IC conversion alleles. This suggests that these genotypes are associated with impaired 11β-hydroxylase activity. A similar association was found between these alleles and plasma 11-deoxycortisol levels following ACTH stimulation in Finnish males (Hautanena *et al* 1998). The SF-1 polymorphism has also been shown to associate with markers of 11β-hydroxylase function in a normotensive population. In this case, the TT group showed increased excretion levels of tetrahydrodeoxycortisol (THS) and tetrahydrodeoxycorticosterone (THDOC), the principle metabolites of S and DOC respectively, compared to the CC group (Connell *et al* 1996). Several aspects of corticosteroid metabolism, including aldosterone excretion rate and markers of 11β-hydroxylase efficiency have been shown to be heritable (Inglis *et al* 1999). Recent studies have also demonstrated that the excretion of THS is heritable (19.4%) and that the -344 T allele is more strongly associated with higher THS levels than the C allele. (Connell *et al* 2004). Importantly, a slightly impaired 11β-hydroxylase function is a consistent finding in subjects with essential hypertension compared to controls (Connell *et al* 1996; de Simone *et al* 1985; Honda *et al* 1977).

How could variations in *CYP11B2* affect 11β-hydroxylase activity? One obvious explanation is that *CYP11B2* polymorphisms are without effect but are in strong linkage disequilibrium (LD) with functional polymorphisms in *CYP11B1*. The *CYP11B1* gene encoding the enzyme 11β hydroxylase is adjacent and homologous to *CYP11B2*. It is possible, even likely, that the -344 and the intron conversion polymorphisms in *CYP11B2* are in linkage with variants in *CYP11B1* and that these are responsible for the observed phenotype of altered 11β-hydroxylase efficiency.

This is supported by experimental evidence of an association between the *CYP11B2* polymorphisms and urinary levels of tetrahydro-deoxycortisol (THS), the principle metabolite of S (Kennon *et al* 2004). Keavney *et al* have shown that the -344C/T and IC polymorphisms in *CYP11B2* are in linkage disequilibrium with two polymorphisms in *CYP11B1*; a CTG to CTA substitution in codon 75 of exon 1 and a G to A substitution in intron 3 (Keavney *et al* 2005). The association with urinary THS levels was found to be strongest with the exon 1 polymorphism, but this is a synonomous base change and so is unlikely to account for the biochemical phenotype of impaired 11β-hydroxylase deficiency. Another study recently found strong linkage disequilibrium between polymorphisms in *CYP11B2* and polymorphisms in *CYP11B1*; a nonsynonymous substitution (T to C) in codon 75 and a G to A substitution in intron 6 (Ganapathipillai *et al* 2005). Using the ratio of urinary THS to total cortisol metabolites (tetrahydroxycortisone (THE) + tetrahydro-11-deoxycortisol (THF) + 5αTHF) as a marker of 11β-hydroxylase function, they reported a decreased 11β-hydroxylase activity in subjects with an increased ARR. The main *CYP11B* haplotypes identified also associated with 11β-hydroxylase efficiency, supporting the hypothesis that markers in *CYP11B1* account for 11β hydroxylase activity *in vivo*.

These findings provide strong evidence that the -344 C/T and IC polymorphisms in *CYP11B2* are in LD with genetic variants in *CYP11B1* that account for the observed phenotype of impaired 11β-hydroxylation. However further fine mapping across the entire *CYP11B* locus is required to identify potential causative variants.

1.16.3 Altered 11β-Hydroxylase Efficiency and Hypertension with a raised ARR

If the -344 C/T and intron conversion polymorphisms identified in *CYP11B2* are in LD with functional variants in *CYP11B1* causing reduced 11β-hydroxylase efficiency, then how does this account for the association with increased aldosterone levels, hypertension and an elevated ARR? It seems plausible that mild long-term deficiency of 11β-hydroxylase activity may result in a resetting of the hypothalamic-pituitary axis by causing a sustained mild increase in ACTH drive to the adrenal cortex to maintain normal plasma cortisol levels (Connell *et al* 2003). If as suggested this mildly impaired 11β-hydroxylase efficiency is genetically determined then it is effectively a minor variant of classical 11β-hydroxylase deficiency (see **section 1.15.2.3**). Lifelong exposure to a slightly enhanced ACTH drive to the adrenal cortex may eventually promote the development of hyperplasia of the zona glomerulosa and zona fasciculata, resulting in increased synthetic capacity for both cortisol and aldosterone. As mentioned previously adrenal gland hyperplasia is a common histologic finding in hypertensive patients at post mortem (Komiya *et al* 1991). ACTH stimulates the expression of a number of genes required for aldosterone synthesis inlcuding StAR, P450scc (*CYP11A*) and P450-21 hydroxylase (*CYP21*) producing a potential increase in aldosterone synthetic capacity (Miller 1988). In support of a role for ACTH in regulating aldosterone is recent research demonstrating that POMC-derived peptides are necessary to develop and maintain normal aldosterone production (Coll *et al* 2004). Although chronic ACTH stimulation has been shown to repress aldosterone synthesis (Oelkers 2000), these studies have used supraphysiological concentrations of ACTH, not equivalent to the

mild and chronic increase suggested here. In ACTH-dependent Cushing's disease, in which there is chronic sustained exposure of the adrenal cortex to ACTH, aldosterone levels are in fact neither suppressed or elevated (Whitworth *et al* 2000). In fact, ACTH increases the expression of a number of genes necessary for aldosterone production emphasizing the potential for enhanced aldosterone synthetic capacity and response to stimuli such as angiotensin II or potassium (Miller 1988). In hypopituitary patients the angiotensin II/aldosterone response is blunted indicating that ACTH may be involved in setting the sensitivity of this relationship (Seifert & Oelkers 1981). Therefore the genetic change in 11β-hydroxylase efficiency may in the long term and in combination with other genetic and environmental factors, promote ACTH dependent adrenal zonal hyperplasia with an increased sensitivity of aldosterone to its main agonist Ang II resulting in hypertension with a raised ARR (Connell *et al* 2003; Freel & Connell 2004). In support of this are early studies demonstrating a good blood pressure response to dexamethasone in patients with essential hypertension, suggesting that ACTH was sustaining production of a hypertensinogenic adrenal steroid (Hamilton *et al* 1979). Also endogenous levels of the adrenal androgen dehydroepiandrosterone sulfate (DHEAS), which is regulated by ACTH, are increased in subjects with hypertension (Schunkert *et al* 1999b).

1.17 Aims

Genetic variation in *CYP11B2* (-344 C/T and intron conversion polymorphisms) has been linked to two key corticosteroid phenotypes in essential hypertension; raised ARR and decreased 11β-hydroxylase efficiency. It is unclear exactly how variation in *CYP11B2* can alter the biochemical function encoded by a neighbouring gene (*CYP11B1*) but the hypothesis that shall be investigated is that the -344 C/T and

Intron conversion variants in *CYP11B2* are in linkage with variants in *CYP11B1* that account for the reduced 11β-hydroxylase efficiency.

The aim of this study was to investigate the way in which genetic variation at the *CYP11B1/B2* locus leads to altered 11β-hydroxylase function through detailed analysis of the pattern of genetic variation across the whole locus. This will hopefully enable the identification of variants across the *CYP11B1/B2* locus that are in LD with the -344 C/T and IC polymorphisms. The functional effects of any variants identified will be tested *in vitro* in order to determine the genetic variation responsible for the observed phenotype of impaired 11β-hydroxylation.

CHAPTER 2

Materials and Methods

2.1 Functional Effects of Mutations in *CYP11B1*

2.1.1 Single Stranded Conformational Polymorphism method

The method of single stranded conformational polymorphism (SSCP) was used in our laboratories to screen for mutations in the coding region of the *CYP11B1* gene by Mrs Christine Holloway and Dr Angela Fisher. Each exon was amplified by PCR from genomic DNA using radiolabelled primers (Oswel DNA Service, Southampton, UK) (**table 1, appendix 2**). Each 25-μL PCR contained 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 12.5 pmol each primer and 1 Unit of thermostable Taq DNA polymerase (Promega Corp., Southampton, UK) and was denatured at 94C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C annealing for 1 min and 72°C extension for 1 min, and a final incubation of 72°C for 7 min. The annealing temperature varied depending on the melting temperature of the primers being used. In most cases the PCR amplicon was greater than 250bp so an appropriate restriction enzyme was used for digestion to produce smaller fragments to ensure increased sensitivity of the SSCP analysis (**table 1, appendix 2**). The PCR products were denatured and separated by electrophoresis (30W, 6h, room temperature) on a non-denaturing 7.5% polyacrylamide gel. Autoradiographs of the gels were produced and the banding pattern of the individual patients compared to determine sequence variants. The presence of any identified mutations were then confirmed by automated sequencing using the ABI Big Dye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA) (see **section 2.2.6**). For exons 2, 3, 4 and 7 it was not possible to selectively amplify *CYP11B1* without *CYP11B2* so if variants were identified in these areas by SSCP the products were subcloned into pGEM easy/T-vector (Promega) prior to sequencing.

In this way, 8 missense mutations were identified in *CYP11B1*, altering the encoded amino acid in exons 1, 2 and 3 at the following locations: R43Q, L83S, H125R, P135S, F139L, L158P, L186V and T196A. Mutant R43Q results in the replacement of the 11β-hydroxylase-specific residue with the corresponding aldosterone synthase-specific residue. To examine the functional effect of these mutations *in vitro* they were recreated by site-directed mutagenesis and transfected into human placental JEG-3 cells (see **section 2.1.4**).

2.1.2 Site-Directed Mutagenesis

The cDNA encoding human 11β-hydroxylase (*CYP11B1*), in the vector $pCMV_4$ was kindly provided by Professor Perrin White. The cDNA insert was 1.5kb and the vector was 4.8kb. *CYP11B1* missense mutations previously detected by SSCP were constructed *in vitro* by site-directed mutagenesis (SDM) of the cloned cDNA. This was performed using the Quick-Change SDM kit (Stratagene Ltd, Cambridge, UK). Mutation of nucleotides encoding amino acids arginine 43, leucine 83, histidine 125, phenylalanine 139, leucine 186, proline 135, leucine 158 and tyrosine 196 was performed using specific sense and antisense mutagenic oligonucleotide primers which were designed individually according to the desired mutations (MWG-Biotech, Ebersberg, Germany) (**table 2, appendix 2**).

2.1.2.1 SDM PCR protocol

SDM was performed according to the kit's standard protocol. Briefly, the component reagents were assembled in a reaction tube and then incubated on a thermal cycler according to the amplification protocol.

Component	Volume
Reaction Buffer (10X)	5µl
DsDNA template (10ng/µl)	4µl
Sense Primer (125ng/µl)	1µl
Antisense Primer (125ng/µl)	1µl
dNTP mix (4mM)	1µl
Nuclease free water (nfH_2O)	37µl
Pfu Turbo DNA Polymerase (2.5U/µl)	1µl
Total volume	**50 µl**

Amplification protocol

1. 95°C	30 seconds	Initial Denaturation
2. 95°C	30 seconds	Denaturation
3. 55°C	1 minute	Annealing
4. 68°C	14 minutes	Extension

Steps 2, 3 and 4 were repeated for 12 cycles.

2.1.2.2 Digestion of parental DNA template

Following temperature cycling, the product was treated with 1 µl (10 U/µl) of the endonuclease DpnI for 1 hour at 37°C. DpnI specifically digests methylated DNA such as that which originates from bacteria. It therefore digests the parental DNA template but leaves the newly synthesized mutation-containing DNA intact.

2.1.2.3 Transformation of Supercompetent Cells

Epicurian Coli XL1-Blue supercompetent cells (50µl) (Stratagene Ltd, Cambridge, UK) were transformed with 4µl of DpnI-treated sample DNA by heat pulsing for 45 seconds in a water bath at 42°C. The cells were then grown in NZY+ broth at 37°C for one hour. The transformation reaction was plated onto LB/ampicillin plates

(50μg/ml) and incubated overnight (>16 hours) at 37°C. Single transformed colonies were used to inoculate overnight incubations, which were shaken at 37°C overnight at 225 rpm in 5ml of LB/ampicillin (1μg/ml) broth.

2.1.2.4 DNA Purification

Plasmid DNA was purified from the overnight incubations using a QIAprep Miniprep Kit (QIAGEN Ltd, West Sussex, UK). The cultured LB was transferred to a 1.5ml Eppendorf tube and centrifuged at maximum speed for 2 minutes in a tabletop centrifuge to pellet the bacterial cells. The cell pellets were resuspended in 250μl Buffer P1. To lyse the cells, 250μl Buffer P2 was added and mixed by gently inverting the tube. 350μl Buffer N3 was added and mixed immediately by inverting the tubes. The samples were centrifuged for 10 minutes at maximum speed in a tabletop microcentrifuge. The supernatants were transferred into a QIAprep Spin Column and centrifuged at top speed for 1 minute. The flow-through was discarded and the column washed by adding 0.5ml Buffer PB and centrifuging at top speed for 1 minute. The wash step was repeated with 0.75ml Buffer PE, and the column was centrifuged for an additional minute to remove residual wash buffer before being placed in a clean 1.5ml microcentrifuge tube. DNA was eluted by adding 50μl nfH_2O to the center of the QIAprep column, letting it stand for 1 minute, and then centrifuging it for 1 minute at top speed. The plasmid DNA minipreparations were stored at 4°C until required for further analysis.

2.1.2.5 Restriction Digestion

Restriction analysis was performed on the plasmid DNA, using the restriction enzyme BamHI, to assess quality and identify vectors containing the appropriate

insert. This enzyme linearizes *CYP11B1* cDNA by hydrolysing it at a single site to produce a fragment of 6.3kb. 2µl of plasmid DNA minipreparation (~ 100ng/µl) was combined with 0.5µl of BamHI, 2µl Buffer J, 0.2µl of BSA and 15.3µl of nfH_2O in a sterile 0.2ml PCR tube. The reaction mixture was incubated at 37°C for 2 hours. The size of digestion product was analysed by electrophoresis of the whole reaction on a 1% agarose gel for 40 minutes at 100 V.

2.1.2.6 Confirmation of Mutations

Colonies of plasmids containing the insert as identified by restriction digestion were sequenced using the automated sequencing method described in Section 2.2.6. The entire insert was sequenced using the primers; V6776, V6777 that bind to the vector and N4019 that binds to the cDNA (see **table 3, appendix 2**), to confirm the presence of the desired mutations and to confirm that no additional mutations were introduced inadvertently.

2.1.3 Large-scale Plasmid Preparations

Large scale plasmid preparations were performed using a Plasmid Midi Kit (QIAGEN Ltd, West Sussex, UK). A single colony from a freshly-streaked selective plate was grown in a starter culture of 5ml LBroth containing the appropriate selective antibiotic for ~8 hours at 37°C with vigorous shaking. The starter culture was diluted 1/100 in selective LBroth and grown at 37°C with vigorous shaking for ~16 hours. The bacterial cells were then harvested by centrifugation at 6000 x g for 15 minutes at 4°C. The bacterial pellet was resuspended in 4ml of Buffer P1. The lysis reaction was initiated by addition of 4ml Buffer P2 and inverting the tube 4-6 times. The solution was then incubated at room temperature for 5 minutes. Next

4ml of chilled Buffer P3 was added, the solution mixed immediately by inversion of the tube 4-6 times and then incubated on ice for 20 minutes. The solution was centrifuged at 16000 x g for 40 minutes at 4°C. A QIAGEN-tip column was equilibriated by applying 4ml of Buffer QBT and allowing the column to empty by gravity flow. The lysate supernatant was applied to the column and allowed to enter the resin. The QIAGEN column was washed with 2 x 10ml Buffer QC. The DNA was then eluted by addition of 5ml Buffer QF. The DNA was precipitated by adding 3.5ml isopropanol to the elute, this was mixed and centrifuged immediately at 16000 x g for 20 minutes at 4°C. The supernatant was discarded and the DNA pellet washed with 2ml of 70% ethanol and centrifuged at 16000 x g for 10 minutes. Again the supernatant was discarded, the pellet air-dried for 2-4 hours and the DNA redissolved in 200µl nfH_2O.

2.1.4 DNA Quantification using PicoGreen method

PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Leiden, Netherlands) is an ultra-sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA). It is more sensitive than the commonly used method of measuring absorbance at 260nm and avoids the interference caused by contaminants commonly found in nucleic acid preparations such as nucleotides, single-stranded nucleic acids or proteins.

Sufficient 1xTE buffer was prepared for the experiment from a 20x stock (Molecular Probes kit). Serial dilutions of samples were pipetted onto a black microassay plate (100µl/well). A DNA standard solution (2µg/µl) was made by diluting 10µl of stock

(100 μg/ml) lambda DNA in 490μl TE. This was used to produce a DNA standard curve in duplicate by loading the following onto a plate:

TE (μl)	DNA Standard (μl)	Final DNA conc
0	100	1mg/ml
50	50	500ng/ml
90	10	100ng/ml
98	2	20ng/ml
100	0	Blank

A working solution of the PicoGreen Reagent was prepared by making a 1:200 dilution of the concentrated stock in TE buffer being careful to maintain the solution in the dark, as PicoGreen is light sensitive. 100μl of this working solution was added to each standard/sample. The plate was then covered in foil to protect it from the light, incubated at room temperature for 5 minutes and then the fluorescence measured using a Wallac Victor 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA, USA).

2.1.5 Transient Transfection system

2.1.5.1 JEG-3 Cells

Human JEG-3 choriocarcinoma placenta cells (American Type Culture Collection) do not normally produce corticosteroids so are suitable for transfection with either of the 11β-hydroxylase or aldosterone synthase genes. Their inability to produce aldosterone or cortisol was confirmed by incubating them with culture medium that did or did not also contain the steroidogenic precursors 11-deoxycorticosterone (DOC) and 11-deoxycortisol (S) respectively and testing the medium for steroid production using HPLC (High-performance liquid chromatography). One main

advantage of these cells is that they have endogenous adrenodoxin, the cofactor essential for the activity of many steroidogenic enzymes. This removed the need to co-transfect with adrenodoxin.

2.1.5.2 JEG-3 cell maintenance

JEG-3 cells were cultured at 37°C in 5% CO_2 in Minimum Essential Medium Eagle's (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 1% sodium pyruvate (100mM) (GIBCO) and 1% penicillin/streptomycin (5000U/ml) (GIBCO).

2.1.5.3 Subculturing procedure

Cells were passaged upon reaching 80-90% confluence. The culture medium was removed and the cells rinsed with phosphate buffered saline solution (PBS). Trypsin-EDTA solution (Sigma-Aldrich) was added to cover the cells and they were incubated at 37°C for 5 minutes to facilitate the dispersal of the cells. 5 ml of complete growth medium was added to inactivate the trypsin and the cells were pelleted at 1500rpm for 5 minutes. The cells were resuspended in complete growth medium and aliquoted into new flasks according to the desired subcultivation ratio.

2.1.5.4 Handling procedure for frozen cells

Cells grown to approximately 90% confluence were typsinized and pelleted as before and resuspended in complete growth medium supplemented with 10% dimethyl sulfoxide (DMSO). Aliquots were transferred to cryovials, which were then frozen gradually over 24 hours in a Nalgene cryo 1°C freezing container. These were then transferred to a liquid nitrogen freezer for long-term storage. When frozen cells were required, they were removed from the liquid nitrogen and the vials thawed by gentle

agitation in a 37°C water bath for 2 minutes. The vial was decontaminated with 70% ethanol. The cells were transferred under sterile conditions to a $75cm^2$ tissue culture flask containing 5ml of prewarmed growth medium and incubated at 37°C in 5% CO_2.

2.1.5.5 Transfection protocol

$1.5x10^5$ JEG-3 cells were plated in 2ml of growth medium on 6-well plates and incubated at 37°C overnight to achieve approximately 80% confluence.

Cell Count

Cell counts were done using a Bright Line Haemocytometer (Sigma-Aldrich). Cells were removed from the flasks using Trypsin-EDTA solution as if being subcultured and spun as before to pellet the cells. The cells were then resuspended in 1ml of complete growth medium. 20µl of resuspended cells were transferred to a sterile centrifuge tube and diluted with 230µl of Hanks Buffer (Sigma-Aldrich). 250µl of Trypan Blue solution (Sigma-Aldrich) was added to stain the cells, inverted to mix and incubated at room temperature for 2-3 minutes. With the coverslip in place, a small amount of Trypan Blue-cell suspension mixture was transferred to both chambers of the haemocytometer. For each chamber of the haemocytometer, the numbers of viable cells (i.e. unstained cells) in the 1mm centre square and the four 1mm corner squares were counted and the mean calculated. Each square of the haemocytometer, with coverslip in place, represents a total volume of $0.1mm^3$ or 10^4cm^3. Since $1cm^3$ is equivalent to approximately 1ml, the subsequent concentration per ml (and the total number of cells) is determined using the following calculation:

Cells/ml = the average count per square x dilution factor x 10^4 x 1 (original vol)

After calculating the number of cells per ml the amount of cell stock needed per well to achieve the correct number of cells (1.5×10^5 cells/well/2ml medium) was determined and the cells plated onto 6-well plates.

Transfection mix

Transfection was carried out using 6µl Fugene (Roche Diagnostics, Mannheim, Germany) and 3µg of wildtype or mutant plasmid in MEM/Eagle's for 24 hours for each well of a 6-well plate. The pGL3 control vector (0.1µg) was added as a reporter gene to measure transfection efficiency. The transfection mix for each well contained:

Component	**Amount/well**
MEM (no additives)	up to 100µl
Fugene	6.0µl
plasmid DNA	3.0µg
pGL3 control plasmid	0.1µg
Total volume	**100µl**

A master-mix was prepared for the wildtype and mutant constructs. The components were added in the order above, the tubes tapped to mix and incubated at room temperature for 30 minutes. 100µl of transfection mix was dropped into the growth medium of each well and the plates swirled to mix. Cells not exposed to any transfection mix were used as a negative control.

2.1.5.6 Steroid Incubation

After 24 hours the transfection medium was removed and replaced with 4 ml of growth medium supplemented with either DOC or 11-deoxycortisol over a range of concentrations (0.1µM, 0.25µM, 0.5µM, 1.0µM and 1.5µM).

Cells were incubated with medium in the absence or presence of substrate for a further 24 hours. All transfections were performed 6 times. The steroid-containing medium was removed for HPLC analysis and the cells lysed for protein measurements (PIERCE, Perbio Science, Northumberland, UK) and luciferase activity (Promega Corp.) (see **section 2.1.6**).

2.1.6 Measurement of Transfection Efficiency

Luciferase activity (Promega Corp.) and protein concentration (Pierce) were measured on cell lysates following the manufacturers' protocols. The luciferase acitivity measured in relative light units (RLU) ranged from $1x10^4$ to $1x10^5$ across all samples and the range of total cell protein levels was 0.5 to 1.2 (arbitrary units). Transfection efficiency was determined by converting protein levels and luciferase activity into RLU per unit of total cell protein. These transfection values were then converted to a proportion of the highest transfection value. The generated correction factors ranged from 0.2 to 2 and the corresponding steroid results were corrected accordingly.

2.1.6.1 Preparation of cell lysates

Cell lysates were prepared using Reporter Lysis Buffer (RLB) (Promega Corp.). After removal of steroid-containing medium, cells were washed with PBS and any residual traces of PBS were then removed. 0.5ml of 1xRLB was added to each well of the 6-well plates. The plates were placed at –70°C for 10 minutes and then thawed at room temperature. The cells were removed by pipetting the RLB over them several times and transferred to a prechilled 1.5ml Eppendorf tube. They were centrifuged at full speed in a microcentrifuge for 2 minutes. Lysates were stored at –

70°C until required. The supernatant was assayed directly for luciferase activity whereas the whole cell lysate was used for determination of protein content.

2.1.6.2 Measurement of Luciferase Activity

Luciferase activity was determined using the Steady-Glo® Luciferase Assay system (Promega Corp.). A 10ng/ml luciferase (Luc) standard stock was prepared using 1x Cell Culture Lysis Reagent (CCLR) (Promega Corp.) containing 1mg/ml BSA. This was used to produce the following concentrations of standard in duplicate:

Vol of stock (µl)	1 x CCLR (µl)	Final Conc (ng/ml)
0	100	0
6.25	93.75	0.625
12.5	87.5	1.25
25	75	2.5
50	50	5
100	0	10

100µl of each standard replicate or sample was pipetted on to a white microwell plate. 100µl of the Steady-Glo Luciferase Assay reagent was added to each standard/sample. This was mixed well and incubated at room temperature for 5 minutes. The light emmitted was measured using a Wallac Victor 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA, USA).

2.1.6.3 Measurement of protein levels

Protein levels were determined with the Pierce BCA (bicinchoninic acid) Protein Assay (PIERCE, Perbio Science, Northumberland, UK). A set of bovine serum albumin (BSA) protein standards were prepared in the range 20–2000µg/ml using the same diluent as the samples (RLB) (Promega Corp.) as follows:

Vial	Vol of Diluent (RLB)	Vol and Source of BSA	Final BSAConc
A	0	300µl Stock	2000µg/ml
B	125µl	375µl Stock	1500µg/ml
C	325µl	325µl Stock	1000µg/ml
D	175µl	175µl of vial B dilution	750µg/ml
E	325µl	325µl of vial C dilution	500µg/ml
F	325µl	325µl of vial E dilution	250µg/ml
G	325µl	325µl of vial F dilution	125µg/ml
H	400µl	100µl of vial G dilution	25µg/ml
I	400µl	0	Blank

25µl of each standard or unknown sample replicate was pipetted on to a clear 96-well plate. A BCA Working Reagent (WR) was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA reagent B. 200µl of the WR was added to each well and mix thoroughly for 30 seconds. The plate was covered and incubated at 37°C for 30 minutes. It was then cooled to room temperature and the absorbance measured at 562nm using a Wallac Victor 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA, USA). The protein concentration was determined from the standard curve.

2.1.7 RNA-Bee Isolation of RNA

2.1.7.1 Cell Lysis

After the removal of the culture medium, cells were lysed directly in the culture dish by the addition of 1ml of RNA-Bee™ RNA isolation reagent (Biogenesis, Poole, UK) to 5 x 10^6 cells. The lysates were passed through a pipette several times to ensure complete lysis and transferred to a 1.5ml Eppendorf tube.

2.1.7.2 Phase Separation

To separate the lysate into aqueous and organic phases, chloroform was added (0.2ml per 1ml of RNA-Bee) and the tube shaken vigorously for 15-30 seconds. The sample

was then stored on ice for 5 minutes before being centrifuged at 12,000 x g for 15 minutes at 4°C. The upper colorless aqueous layer was carefully removed and transferred to a clean tube without touching the lower blue phenol-chloroform phase containing DNA and protein.

2.1.7.3 RNA Precipitation

Isopropanol (0.5ml) was added and the sample stored at room temperature for 5-10 minutes. It was then centrifuged at 13,000 x g for 5 minutes at 4°C. The RNA precipitate, which is often not visible before centrifugation, forms a white/yellow pellet at the bottom of the tube.

2.1.7.4 RNA Wash

The supernatant was removed and the RNA pellet washed with 75% ethanol in a volume equal to the starting amount of RNA-Bee. The tube was vortexed to dislodge the pellet from the side of the tube and centrifuged for 5 minutes at 7,500 x g at 4°C.

2.1.7.5 RNA Solubilization

The RNA pellet was briefly air-dried (5-10 minutes) to remove residual traces of ethanol but not allowed to dry completely as this greatly reduces its solubility. Each RNA sample was dissolved in 20µl nfH_2O by passing the solution through a pipette tip several times then stored at –70°C.

2.1.7.6 DNase Treatment

Each RNA solution was treated with DNA-free™ (Ambion, Huntingdon, UK) to remove any DNA contamination. 0.1 vol of 10 x DNase I buffer and 1µl (2 units) of DNase I was added to each sample. The samples were mixed and incubated at 37°C

for 20-30 minutes. 5µl of DNase Inactivation reagent was then added and the samples mixed well. They were then incubated for 2 minutes at room temperature, flicking the tube once during the incubation to mix. The samples were centrifuged at 10,000 x g for 1 minute to pellet the DNase inactivation reagent and the RNA transferred to a new tube. The RNA was then stored at –70°C.

2.1.8 RNA Quantification using RiboGreen® method

The RiboGreen® RNA quantitation kit (Molecular Probes, Leiden, Netherlands) allows reliable detection of RNA in solution, with a sensitivity that is orders of magnitude greater than UV absorbance readings.

2.1.8.1 Sample Preparation

A 1 x Tris-HCl ethylenediaminotetraacetic acid (EDTA) buffer working solution (1 x TE) was prepared by diluting 20 x TE buffer with nfH_2O. The RNA sample solutions were diluted 2000-5000 fold in 1 x TE. 100µl of each diluted RNA sample was loaded into a well of a 96-well black microplate (Nalge Nunc International, Hereford, UK).

2.1.8.2 RNA standard curve

The ribosomal RNA standard, provided at 100µg/ml in the RiboGreen kit, was diluted 50-fold in 1 x TE to make a 2µg/ml working solution. This was diluted in the black microplate as shown:

Vol(μl) of TE	Vol (μl) of 2 μg/ml RNA Stock Solution	Vol (μl) of Diluted RiboGreen Reagent	Final RNA Concentration
5	100	100	1000ng/ml
50	50	100	500ng/ml
90	10	100	100ng/ml
98	2	100	20ng/ml
100	0	100	Blank

2.1.8.3 Reagent Preparation

An aqueous working solution of the RiboGreen reagent was prepared by diluting an aliquot of the concentrated dye solution stored in anhydrous dimethylsulfoxide (DMSO) into 1 x TE using sterilized polypropylene plasticware, 200-fold. 100μl of the working solution was loaded into each well and mixed with the RNA sample solution. The plate was foil-wrapped to protect it from light and incubated for approximately 5 minutes at room temperature.

2.1.8.4 Fluorometry counting

The plate was loaded manually on to a Wallac Victor 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA). The template layout within the Workstation software was configured to describe the group and type of each well on the virtual microplate. The machine measured the fluorescent emission of the samples at wavelength 535nm after excitation at 485nm. RNA concentrations were then calculated for each sample, compensating for the dilution factor of each diluted sample.

2.1.9 Real-time quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR)

One-step real-time QRT-PCR was performed using the LightCycler System (Roche Diagnostics, Mannheim, Germany) and the LightCycler RNA Master Hybridization Probes kit (Roche Diagnostics). A homologous mRNA standard for human *CYP11B1* was prepared from commercially available adrenal cDNA using specific primers, hB1sense (exon 8) and hB1antisense (exon 9) (MWG-Biotech, Ebersberg, Germany) (**table 4, appendix 2**), following the protocol previously published for rat standard (Ye *et al* 2003). The same primers were used in the RT-PCR reaction. *CYP11B1* sequence-specific hybridization probes were designed and synthesized by TIB Molbiol (Berlin, Germany). These consisted of a fluorescein probe and a LightCycler Red probe (**see table 4, appendix 2**).

A master mix solution was prepared in a 1.5ml Eppendorf tube on ice by adding the reaction components in the order given below. The standard reaction required to perform a single colour detection takes place in a 20µl volume. The amount in the "volume" column was multiplied by the number of reactions to be cycled, plus one additional reaction.

Component	**Volume**	**Final conc.**
H2O, PCR grade	6.2µl	
$Mn(OAc)_2$ stock solution, 50mM	1.3µl	3.25mM
B1 Fluorescin (FL)	0.4µl	0.2µM
B1 LightCycler Red705 dye (LC)	0.4µl	0.2µM
Primer HB1 sense	1.0µl	0.5µM
Primer HB1 antisense	1.0µl	0.5µM
LightCycler RNA Master Hybridization Probes, 2.7 x conc	7.5µl	1 x
Total volume	**19µl**	

This was mixed gently and 19µl of master mix pipetted into a precooled LightCycler Capillary (Roche Diagnostics, Mannheim, Germany). 1µg (at 1µg/µl concentration) of the RNA template was added to the capillary. A control reaction was also constructed by replacing the RNA with nfH_2O (water blank). The capillaries containing the samples were placed in a holder and centrifuged at 700g for 5 seconds in a standard benchtop centrifuge (Roche Diagnostics) before being placed in the rotor of the LightCycler Instrument (Roche Diagnostics). The cycle programs were performed in the order:

1) Reverse Transcription (RT)

2) Denaturation

3) Amplification

4) Cooling

The cycle parameters were as follows:

Cycle Program Data	**RT**	**Denaturation**	**Amplification**			**Cooling**
Cycles	1	1	45			1
Analysis Mode	None	None	Quantification			None
Temperature Targets	S1	S1	S1	S2	S3	S1
Target Temperature (°C)	61	95	95	58	72	40
Incubation time	20 min	30 s	1 s	15 s	13 s	30 s
Temperature Transition Rate (°C/s)	20	20	20	20	2	20
Secondary Target Temperature (°C)	0	0	0	0	0	0
Step Size (°C)	0	0	0	0	0	0
Step Delay (Cycles)	0	0	0	0	0	0
Acquisition Mode	None	None	None	Single	None	None

The annealing temperature of the *CYP11B1* probes was 58°C. Fluorescent data are collected during the single acquisition phase. 1ml aliquots of sequential tenfold mRNA standard dilutions were used to construct a standard curve. The sample copy

number was extrapolated from the standard curve at the base of the sample's crossing point. Each sample was measured three times and a water blank was included in each run.

2.2 Screening of *CYP11B1* and *CYP11B2* genes for Polymorphisms in a Normotensive Population

2.2.1 Study Subjects

Normotensive subjects were selected from a WHO survey of cardiovascular risk factors (MONICA IV), which was a random sample of the north Glasgow patient lists of 30 general practitioners (Fraser *et al* 1999). Study subjects completed a health questionnaire and attended for a structured medical examination to assess overall cardiovascular risk. Approval was obtained from the appropriate ethics committee. The study group consisted of 238 male and 201 female subjects aged 36 to 57. They were all normotensive with a fairly narrow blood pressure range within the group. These subjects had already been genotyped for, and were selected according to, the *CYP11B2* -344 C/T and intron Conversion (IC) variants. The IC alleles are designated wildtype (Wt) for the *CYP11B2* intron 2 sequence or conversion (Con) when the intron 2 sequence of *CYP11B1* has been introduced.

2.2.2 Polymerase Chain Reaction amplification of CYP11B1

Subjects were selected who were homozygous at both the -344 C/T and IC loci. Twenty-six subjects were selected for genetic analysis; TT/ConCon (10), CC/WtWt (10) and TT/WtWt (6). The aim was to have ten subjects in each genotype group but

only six of the 439 subjects were TT/WtWt and none were CC/ConCon, which reflects the population frequencies of these haplotypes.

Miss Donna M Wilkinson and Dr. E. Marie Freel were responsible for the amplification and sequencing of the *CYP11B2* gene within these subjects. The amplification of *CYP11B2* was split into 3 separate PCRs: (i) from the 5' region to intron 4 (~5Kb); (ii) exon 5 to exon 9 (~3Kb); (iii) the 3'UTR (~1.5Kb). The sequences of the primers (MWG-Biotech, Ebersberg, Germany) used for these reactions are listed in **table 5, appendix 2**.

The *CYP11B1* gene was amplified in two reactions encompassing:

i) the 5' promoter region to exon 9 (~7kb)

ii) the 3'UTR (~750bp).

HPLC-purified synthetic oligonucleotides were designed using the published sequences of *CYP11B1* and *CYP11B2* (see **appendix 1**) and obtained from a commercial source (MWG-Biotech, Ebersberg, Germany). Primers were generally between 20-24 base pairs and had approximately 50% GC ratios (see **table 5, appendix 2**). The melting temperature (T_m) of each of the primers was approximately 60°C.

2.2.3 PCR Protocols

Both PCRs were performed using the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany). This system is composed of a unique enzyme mix with proof-reading capability to avoid the unwanted introduction of PCR-generated mutations.

5'UTR to Exon 9 PCR

Two separate reaction master-mixes were prepared in sterile 1.5ml Eppendorf tubes on ice.

MIX 1

Component	**Volume/Tube (µl)**	**Final Concentration**
Template DNA	1.0	(20-100ng/µl)
dATP (10mM)	0.5	200µM
dCTP (10mM)	0.5	200µM
dGTP (10mM)	0.5	200µM
dTTP (10mM)	0.5	200µM
Sense primer, 10µM (B1 5'UTR)	0.75	300µM
Antisense primer, 10µM (B1 Exon9 AS)	0.75	300nM
nfH_20	8.0	—
Total	**12.5µl**	

MIX 2

Component	**Volume/Tube (µl)**	**Final Concentration**
10X Buffer (15mM $MgCl_2$)	2.5	1 x (1.5mM $MgCl_2$)
Expand High Fidelity Enzyme mix	0.75	2.6 U/reaction
nfH_20	9.25	—
Total	**12.5µl**	

A 'blank' reagent control was prepared which contained all the necessary components for PCR but replaced template DNA with nfH_20. The total reaction volume was 25µl. The reaction mixes were subsequently combined on ice in single 0.2ml thin-walled amplification tubes (ABgene, Surrey, UK) before being placed in a 96-well AlphaTM sample block powered by the PTC-225 DNA Engine Tetrad® Cycler with heated lid (MJ Research, Waltham, MA, USA). Amplification cycles and reaction conditions were as follows:

Amplification Protocol

1. 94°C	2 minutes	Denaturation
2. 94°C	15 seconds	
3. 65°C	30 seconds	Amplification (10 cycles)
4. 68°C	8 minutes	
5. 94°C	15 seconds	
6. 65°C	30 seconds	Amplification (20 cycles)
7. 68°C	8 minutes + 5sec/cycle	
8. 72°C	7 minutes	Extension

3'UTR PCR

Two separate reaction master-mixes were prepared on ice, as above, the only difference being the primers used (see **table 5, appendix 2**):

- Sense primer (M6635)
- Antisense primer (B13'UTR+500)

Again the total reaction mix was 25µl. The reaction mixes were combined and kept on ice before being placed in an Alpha™ sample unit of a DNA Engine Tetrad® Peltier thermal cycler. The amplification cycles and reaction conditions for this reaction were as follows:

Amplification Protocol

1. 94°C	2 minutes	Denaturation
2. 94°C	15 seconds	
3. 55°C	30 seconds	Amplification (10 cycles)
4. 72°C	1 minutes	
5. 94°C	15 seconds	
6. 55°C	30 seconds	Amplification (20 cycles)
7. 72°C	1 minutes + 5sec/cycle	
8. 72°C	7 minutes	Extension

2.2.4 *Determination of PCR products*

1g of agarose (Invitrogen, Paisley, UK) was added to 1 x TAE buffer (100ml), mixed and heated in a 650W microwave oven for 60 seconds. After 3 minutes of cooling, 1µl of ethidium bromide (10mg/ml) was added and mixed in. The agarose was then poured into gel moulds with Teflon combs and allowed to set for 20 minutes before combs were removed. Gels were submerged in 1 x TAE buffer in standard electrophoresis tanks (Wide Minis Sub Cell Agarose gel apparatus) (Bio-Rad Laboratories, Hercules, CA, USA). 10µl of PCR product was loaded with 5µl of loading dye onto the agarose gel. The DNA molecular weight marker Hyperladder I (Bioline, London, UK) was also loaded in a separate well. The gel was then resolved at 95V for 40 minutes. DNA bands were visualized under a UV light.

2.2.5 *PCR clean-up*

Prior to sequencing, PCR products were cleaned to remove excess dNTPs and primers, using the enzymes Shrimp Alkaline Phosphatase (SAP) and Exonuclease I (ExoI). SAP removes the phosphate groups from excess dNTPs remaining from the PCR reaction; ExoI digests the single stranded PCR primers into dNTPs and their phosphate groups are removed by the SAP. 15µl of PCR product was transferred to sterile, thin walled PCR (0.2ml) tubes. 1µl SAP (1U/µl) and 0.5µl ExoI (20U/µl) were added and the samples incubated at 37°C for 30 minutes followed by 10 minutes at 80°C on the Tetrad® thermal cycler.

2.2.6 *Automated Cycle Sequencing*

Automated sequencing was performed using the ABI Big Dye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems). The technique is an adaptation of the

dideoxy termination method of sequencing. The primers used for sequencing the entire *CYP11B1* gene are shown in **table 6, appendix 2**.

Sequencing reaction set-up

Sequencing reactions were set up in 96-well plates containing the following:

Template (50-100ng)	x µl
Sequencing Primer (3.2pmol/µl)	1.0µl
Ready Reaction mix	0.5µl
Sequencing Buffer	3.8µl
Distilled Water (up to final vol 20µl)	x µl

After setting up gradients of template volumes it was found that for the 5' to exon 9 (7kb) PCR, the optimal volume of template for sequencing was 2µl (~ 100ng) whereas only 1µl (~ 50ng) was required for the 3' UTR (750bp). The sequencing reactions were then incubated on an Alpha™ sample unit of a DNA Engine Tetrad® Peltier thermal cycler as follows:

Reaction conditions

1. 96°C	45 seconds	
2. 50°C	25 seconds	(These 3 steps are repeated 25 times.)
3. 60°C	4 minutes	

Sequencing reaction clean-up

Keeping the sequencing reactions in the 96-well PCR plates, a sodium acetate/ethanol precipitation was performed as follows.

A solution of sodium acetate (NaOAc) in 95% ethanol (EtOH) in the ratio 2µl 3M NaOAc: 50µl 95% EtOH was prepared and 50µl of this solution added to each sequencing reaction. The plate was then sealed and inverted a few times to mix. The sample were incubated at room temperature for 15 minutes to precipitate the

extension products. The plate was centrifuged for 30 minutes at 6000 x g and the supernatant immediately discarded. The plate was centrifuged again whilst inverted on a paper towel for 30 seconds at 500 x g to remove all residual traces of supernatant. 150µl of 70% ethanol was added to each well and the plated sealed and inverted again before being centrifuged at 6000 x g for 10 minutes. Again the supernatant was removed by centrifuging the inverted plate.

2.3 Genotyping of *CYP11B1* 5'UTR Polymorphisms in a Hypertensive Population

Two novel polymorphisms within the 5' UTR of *CYP11B1* (-1889 G/T and -1859 A/G) were identified during sequencing of *CYP11B* locus within the normotensive cohort. The entire MRC BRItish Genetics of HyperTension (BRIGHT) study population (n = 4020) were genotyped for these polymorphisms to assess their frequency and phenotypic associations in a large hypertensive population. A random subgroup of 512 unrelated subjects were selected for further analysis of corticosteroid metabolites.

2.3.1 Study Subjects

The MRC BRItish Genetics of HyperTension (BRIGHT) study is a large multicentre investigation into the genetic basis of hypertension comprising > 2500 affected sibling pairs and trios. Hypertensive subjects were recruited from primary care with entry to the study being dependent on a pre-treatment blood pressure of >145/95mmHg (mean of 3 readings) which lies within the top 5% of the population BP distribution in the UK. Subjects with a body mass index (BMI) >30 were excluded from the study. Full details of the recruitment strategy have been published

elsewhere (Caulfield *et al* 2003). The local ethics committees of the partner institutes granted ethical approval for the study and fully informed written consent from all of the participants was obtained.

2.3.2 Nested PCR Protocol

In order to increase specificity of the genotyping, a nested PCR (i.e. PCR amplification of the PCR product using specific primers) was performed. The initial reaction amplified a 2kb region specific to *CYP11B1* and the second reaction used this product as a template to specifically amplify the region of interest (~350bp). In both reactions, a heat-activated thermostart Taq polymerase (ABgene, Surrey, UK) was used. The sense and anti-sense primers (MWG-Biotech, Ebersberg, Germany) used in both reactions are outlined in **table 7, appendix 2**.

PCR Amplification 1

Component	**Volume (µl)**	**Final Conc**
Buffer (10x)	250	1x
$MgCl_2$ (25mM)	150	1.5mM
dNTPs	500	200µM each
B15'UTR (sense)	100	400nM
B1prom-260 (antisense)	100	400nM
Thermostart Taq polymerase	25	1.25U
nfH_2O	875	—
Total	**2000**	

This was mixed and 20µl of master mix added to 5µl of BRIGHT template DNA (5ng/µl), which had been pre-plated onto 96-well PCR plates. The amplification reaction was performed on a 96-well Alpha™ sample block powered by the PTC-225 DNA Engine Tetrad® Cycler with heated lid (MJ Research, Waltham, MA, USA). The cycling parameters were as follows:

1. 95 °C	15 minutes		
2. 95 °C	30 seconds	}	x 35
3. 60 °C	30 seconds		
4. 72 °C	3 minutes		
5. 72 °C	7 minutes		

Steps 2, 3 and 4 were repeated 35 times. The PCR products were then diluted 1:10 in nfH_2O and 1µl used as a template for a second PCR reaction. Again a mastermix was prepared on ice, this time in a 5ml universal tube:

PCR Amplification 2

Component	**Volume (µl)**	**Final Conc**
Buffer (10X)	250	1x
MgCl2 (25mM)	150	1.5mM
dNTPs	500	200µM each
B15'7-32 (sense)	100	400nM
B1393-369 (antisense)	100	400nM
Thermostart Taq polymerase	12.5	0.625U
nfH_2O	1287.5	—
Total	**2400**	

This was mixed and 24µl of master mix pipetted into the wells of a fresh 96-well PCR plate. 1µl of a 1:10 dilution of PCR amplification mix 1 was then added to serve as the template. The amplification conditions were as the same as before, except that the number of cycles was reduced to 20.

PCR clean-up

The PCR products were then cleaned using a NucleoFast® 96 PCR clean-up system. The NucleoFast® 96 PCR clean-up system is designed to retain PCR products on an ultrafiltration membrane under vacuum whilst contaminants (dNTPs, primers and

salts) are filtered to waste. The PCR samples (25µl) were centrifuged briefly before being transferred to a NucleoFast® 96 PCR plate. The plate was placed on a vacuum manifold and a vacuum was applied until the samples had passed into the plate. To wash the samples, 100µl of nfH_2O was dispensed into each well and the vacuum applied until the water had passed through the membrane. 50µl of nfH_2O was pipetted directly on to the membrane of the NucleoFast® plate. The water was pipetted up and down several times before being transferred to a clean 96-well PCR plate and stored at 4°C until required.

Determination of genotypes

Each of the samples were sequenced by the automated sequencing method mentioned in **section 1.2.6**, using 6µl of NucleoFast PCR product and the primer B1prom-1750(as) (**table 6, appendix 2**).

2.3.3 Urinary Corticosteroid Metabolite Measurements

Miss Mary C. Ingram performed the urinary corticosteroid measurements. 24-Hour urine samples were collected in plain plastic containers without preservative. Aliquots of urine were stored at –20°C. Excretion rates of steroid metabolites were measured by gas chromatography/mass spectrometry using the method of Shackleton (Shackleton 1993) with minor modifications.

2.4 Analysis of *CYP11B1* 5'UTR polymorphisms *in vitro*

2.4.1 Luciferase Reporter Gene System

The effects of the 2 polymorphisms identified in the 5' region of *CYP11B1* were investigated using a reporter gene system. The *CYP11B1* 5' region (1937bp) was

cloned into the pGL3 Basic vector (Promega Corp.) by Mrs Christine Holloway. This vector lacks a eukaryotic promoter sequence and so expression of luciferase activity in cells transfected with this plasmid depends on insertion and proper orientation of a functional promoter upstream of the luciferase gene. This construct (pB1–1937) was used as a template for production of each of the variants to be studied.

2.4.1.1 SDM

The -1889 G/T and -1859 A/G polymorphisms were recreated by SDM following the method described in **section 2.1.1**. Specific sense and antisense mutagenic oligonucleotide primers were designed individually according to the desired mutations (MWG-Biotech, Ebersberg, Germany) (**table 8, appendix 2**). These were designed against the template sequence (**appendix 1**) to produce the alternative alleles for each polymorphism. Once these had been created, one construct was used as a template for the alternative pair of primers so that all 4 combinations of the alleles were created. The entire insert in each case was sequenced to confirm the presence of the desired mutations and that no unwanted base changes had been incorporated.

2.4.1.2 Y-1 cell maintenance

To analyze the *CYP11B1* 5' UTR reporter gene expression, cells were required that possess all the necessary transcription factors to bind the *CYP11B1* 5'UTR or regulatory region and transcribe the luciferase gene. Therefore an adrenal cell line was used.

Y-1 mouse adrenal cells (American Type Culture Collection) were cultured at 37°C in 5% CO_2 in F-12K Nutrient Mixture, Kaighn's Modification (GIBCO)

supplemented with 15% horse serum (GIBCO), 2.5% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (5000U/ml) (GIBCO). The subculturing and handling procedures were as reported for the JEG-3 cells in **sections 2.1.4.2** and **2.1.4.3.**

2.4.1.3 Transfection Protocol

Cells were plated at $3.5x10^5$ cells/well in 6-well plates and incubated for 24 hours prior to transfection. Transfection was carried out using 6µl Fugene (Roche Diagnostics, Mannheim, Germany) and 1µg of wildtype or mutant plasmid in MEM/Eagle's (2ml) for 42 hours. The pSV-β-Galactosidase (pSV-β-Gal) control vector (0.04µg) (Promega Corp.) was added as a marker of transfection efficiency. Cells transfected with the pGL3 basic vector not containing any insert and cells not exposed to any transfection mix were used as negative controls.

2.4.1.4 Agonist stimulation

Following transfection for 42 hours, the transfection medium was removed and replaced with 2ml of growth medium containing agonist: either ACTH (1µM) or Forskolin (10µM) (Sigma-Aldrich). Cells were treated with agonist for 6 hours.

The cells were then lysed and luciferase activity measured as in **section 1.1.5.2** (Promega Corp.). Protein (PIERCE) and β-galactosidase activity (Promega Corp.) measurements were used to determine transfection efficiency and cell number and the luciferase results were corrected accordingly.

2.4.2 Measurement of transfection efficiency

Preparation of cell lysates

Cells lysates were prepared as in **section 2.1.5.1** using 250 µl of RLB.

Measurement of protein levels

Protein levels were determined using the BCA Protein Assay (PIERCE) following the protocol described in **section 2.1.5.3**.

2.4.2.1 Measurement of β-Galactosidase Activity

β-Galactosidase activity was measured using the Tropix® Galacto-Light Plus™ chemiluminescent reporter gene assay system (PE Applied Biosystems). β-Galactosidase activity was determined in cell extracts transfected with the pSV-β-Gal reporter gene. A lyophilized β-galactosidase standard (Sigma-Aldrich) was reconstituted to 1mg/ml in 0.1M sodium phosphate (pH 7.0) containing 0.1% BSA (Sigma-Aldrich). This was diluted to a 1ng/µl stock solution in lysis solution (100mM potassium phosphate, 0.2% Triton X-100) containing 0.1% BSA with a pH of 7.8. Standards (20µl) in the range 4 to 20ng as well as a 'blank' consisting of only lysis solution, were plated in duplicate alongside each of the samples (20µl) on a white 96-well plate. The chemiluminescent Galacton-Plus® substrate (100 x concentrate) was diluted 1:100 with Reaction Buffer Diluent and 70µl of the resulting reaction buffer added to each well. Samples were then mixed and incubated at room temperature for 1 hour. During this time the substrate Galacton-Plus (a 1,2-Dioxetane) becomes cleaved by the enzymatic activity of the β-galactosidase contained in the sample, as the conditions (pH 7.8) are optimal for

bacterial β-galactosidase. The reactions were terminated by the addition of 100μl Accelerator II, which increases the pH to >12, triggering light emission. Light emission was measured immediately at 475nm using a Wallac Victor 1420 Multilabel Counter (PerkinElmer Life Sciences, Boston, MA, USA).

CHAPTER 3

Functional Effects of Mutations within the 11β-Hydroxylase (*CYP11B1*) gene

3.1 Introduction

Recent studies showing that up to 15% of unselected patients with hypertension have a raised aldosterone to renin ratio suggest that altered regulation of aldosterone production may be a common factor in essential hypertension (Fardella *et al* 2000; Gordon *et al* 1994b; Lim *et al* 1999b; Loh *et al* 2000; Rayner *et al* 2000). Two common variants (-344 C/T and intron conversion) in the aldosterone synthase (*CYP11B2*) gene have previously been associated not only with aldosterone levels and hypertension but also with markers indicating an impaired 11β-hydroxylase efficiency, the enzyme product of the *CYP11B1* gene (see **section 1.16**). However, *in vitro* studies suggest that the -344 C/T has no effect on gene transcription, suggesting that it is not the functional variant and may therefore be in linkage disequilibrium with an as yet unidentified functional variant, possibly in *CYP11B1* (Bassett *et al* 2002).

3.2 Aims

The aims of this study were to identify polymorphisms in *CYP11B1* that associate with hypertension and a raised ARR and therefore might contribute to the genetic component of these phenotypes. The functional significance of any variants identified was then investigated *in vitro*.

3.3 Methods

3.3.1 Subjects

Four groups of subjects, normotensive controls, unselected essential hypertensive subjects or hypertensive subjects with either a high ($\geq$ 750) or low ARR ($\leq$ 200) were screened for mutations. Each group comprised 40 subjects. The normotensive controls were selected randomly from the Scottish Adult Twin Study (Inglis *et al* 1999). This study contained data from 146 pairs of adult twins, aged 31-83 years. Only one twin from each pair was selected for mutation detection. The selected controls had no previous history of hypertension; in each subject in this group, blood pressure was < 140/90 mmHg at a screening visit. The unselected essential hypertensive subjects were chosen at random from a total of 138 patients recruited from the Blood Pressure Clinic at the Western Infirmary, Glasgow (Davies *et al* 1999). All were aged <64 years and had high blood pressure confirmed by 3 independent measurements of >160/90 before initiation of treatment. The low and high ARR subjects were randomly selected from a total of 375 patients referred to the Tayside Hypertension Clinic at Ninewells Hospital, Dundee (Lim *et al* 2002b). They were aged 18-80 years and all had blood pressures >160/90 mmHg, confirmed over a period of at least 3 months before referral. Both high and low ARR groups had mean aldosterone levels within the normal range with no significant difference between the groups. Therefore, renin levels largely determined the differences in ARR (see **table 3.1**).

3.3.2 Identification of Mutations

The coding regions of *CYP11B1* were initially screened by SSCP (single-stranded conformation polymorphism), and the presence of any mutations confirmed by direct sequencing by Mrs Christine Holloway and Dr Angela Fisher. The effect of each of these on enzyme activity was determined *in vitro*.

3.3.3 Investigation of enzyme activity in vitro

Each of the mutants was prepared by site-directed mutagenesis using the Quick-Change site-directed mutagenesis kit (Stratagene Ltd, Cambridge, UK) and the $pCMV_4$-B1 plasmid as a template (**section 2.1.1**). Large scale purified preparations of the $pCMV_4$-B1 wildtype, each of the mutants and the pGL3 control plasmid were made, analysed by restriction digestion and sequenced (**sections 2.1.1, 2.1.2, 2.2.6**). Transient transfection was carried in JEG-3 cells (**section 1.1.4**). Cells were incubated with or without substrate (S or DOC) at a range of concentrations for 24 hours. The incubation medium was then removed for steroid analysis by HPLC. Cells lysates were prepared for protein and luciferase measurements to determine cell number and transfection efficiency and steroid results were corrected appropriately (**section 2.1.5**). RNA was extracted from cells transfected with the wildtype gene or each of the mutant constructs for quantitative RT-PCR to compare *CYP11B1* RNA transcript levels (**sections 2.1.6 to 2.1.8**).

3.3.4 SF1 (-344 C/T) genotyping

The subjects were already genotyped for the SF1 (-344 C/T) polymorphism by PCR and *Hae*III restriction digestion following the conditions previously described (Davies *et al* 1999).

3.3.5 Statistical Analysis

Although the sample sizes for the transfection experiments were relatively small (n = 6) they all passed the Kolmogrov-Smirnov normality test indicating a Gaussian distribution and that parametric testing was suitable. Statistical comparison of mean steroid levels over the range of substrate concentrations was accomplished by 2-way analysis of variance (ANOVA). Comparison of fold differences compared to wildtype for each of the mutants at a single substrate concentration was accomplished using a 2-sample t test. The mRNA expression rates were not normally distributed even when log-transformed and so were analyzed by the Mann-Whitney nonparametric test. For all analyses, $p < 0.05$ was required for statistical significance.

3.3.6 Ethics

The local ethics review commitees approved the patients' recruitment and subsequent genetic studies.

3.3.7 Modelling

The protein structure of 11β-hydroxylase ($P450_{11\beta}$) has yet to be determined and so, to give some idea of where the mutant amino acids lie in relation to important

functional regions of the protein, the best 3D model possible was produced using the information currently available.

The P450 superfamily proteins all have a similar folded structure (Nelson and Strobel 1989). This enables tentative modelling of unknown proteins on the basis of those crystal structures that have been determined. Krone *et al* confirmed that $P450_{11\beta}$ follows the general topography of the P450 superfamily, and out of the known crystal structures, mammalian CYP2C5 had the highest structural similarity (Krone et al 2005). Therefore, the structure of CYP2C5 (PDB accession code 1DT6) was used as a template for the 3D model of $P450_{11\beta}$.

The amino acid sequences of $P450_{11\beta}$ (*CYP11B1*) and 21-hydroxylase (CYP2C5) were aligned using the MultiAlin multiple sequence alignment (Corpet 1988). Human 11β-hydroxylase contains 503 amino acids and mammalian (rabbit) 21-hydroxylase contains 473 amino acids. $P450_{11\beta}$ has 24 residues at its N-terminus that constitute the mitochondrial targeting sequence. These are not present in 21-hydroxylase as it is a microsomal P450; the alignment begins after this targeting sequence.

The structure of CYP2C5 was imported into QUANTA (Accelyrs, San Diego, CA, USA) and according to the sequence alignment, amino acid residues were exchanged in the template. Refinement and energy minimization were carried out by REFMAC5 (Murshudov *et al* 1997) and the model validated by PROCHECK (Laskowski *et al* 1993).

3.4 Results

3.4.1 Subjects

Both the low and high ARR groups had mean aldosterone levels within the normal range. The mean aldosterone level was 406 ± 54.29pmol/l for the low ARR (≤ 200) group and 503 ± 40.26pmol/l for the high ARR (≥ 750) group (see **table 3.1**). Therefore, differences in their plasma renin levels accounted for the differences in ARR. The renin level for the low ARR group was 4.81 ± 0.7 compared to 0.23 ± 0.03 for the high ARR group.

	Aldo (pmol/l) Mean ± SEM	PRA (ngAl/ml/hr) Mean ± SEM	ARR Mean ± SEM
ARR ≤ 200	406 ± 54.29	4.81 ± 0.7	124 ± 8.14
ARR ≥ 750	503 ± 40.26	0.23 ± 0.03	4089 ± 831.54

Table 3.1. Mean plasma aldosterone (Aldo) levels and renin activity (PRA) for the hypertensive groups stratified by ARR

3.4.2 Mutation Detection

No mutations were identified that differed in frequency between the four groups of subjects. However, eight missense mutations were identified in *CYP11B1*, which alter the encoded amino acid. These were located in exons 1, 2 and 3: R43Q, L83S, H125R, P135S, F139L, L158P, L186V and T196A. Mutant R43Q results in the replacement of the 11β-hydroxylase-specific residue with the corresponding aldosterone synthase-specific residue. Each of the mutations were identified in individual subjects apart from L158P, which was identified in two subjects. Mutants P135S, F139L and T196A were identified in the same subject (see **table 3.2**).

Exon	Mutant	Base change	Amino acid change	Group
1	R43Q	CGG - CAG	Arginine - Glutamine	ARR > 750
2	L83S	TTG - TCG	Leucine - Serine	Unselected Hypertensive
3	H125R	CAC - CGC	Histidine - Arginine	Unselected Hypertensive
3	P135S*	CCT - TCT	Proline - Serine	Control
3	F139L*	TTC - CTC	Phenylalanine - Leucine	Control
3	L158P	CTC - CCC	Leucine - Proline	ARR < 200/ Control
3	L186V	CTG - GTG	Leucine - Valine	Unselected Hypertensive
3	T196A*	ACC - GCC	Threonine - Alanine	Control

Table 3.2 *CYP11B1* Missense Mutations

All mutations were identified in individual subjects, apart from L158P, which was identified in 2 subjects.

* *All of these mutants were identified within the same subject*

3.4.3 Restriction Digestion of Plasmids

Plasmid DNA was digested using the restriction enzyme BamHI to assess quality and the presence of an insert of appropriate size. This enzyme linearizes *CYP11B1* cDNA by hydrolysing it at a single site to produce a fragment of 6.3kb. BamHI digests are shown in **figure 3.1**.

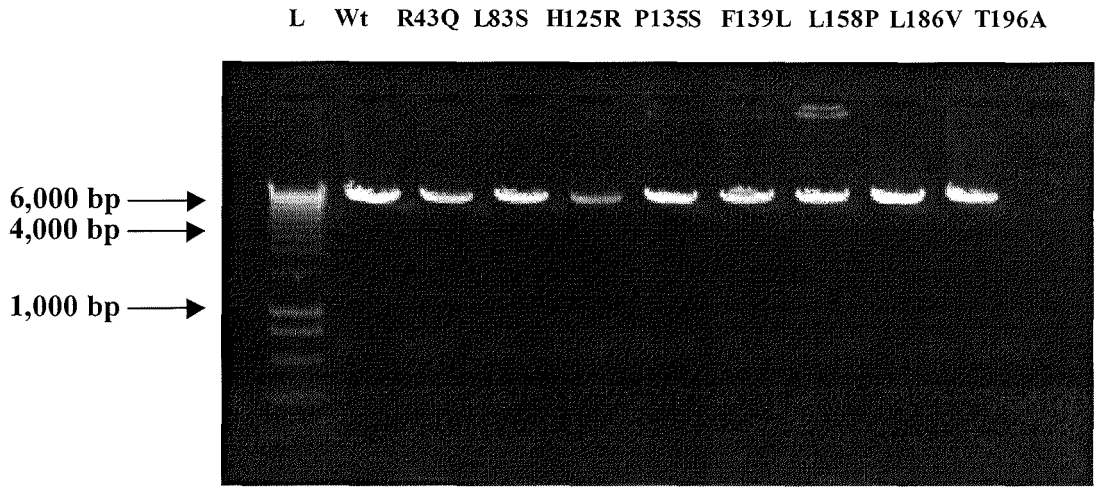


Figure 3.1 BamHI digests of pCMV4-B1 wild-type and mutant plasmids

Analysed on a 1% agarose gel stained with ethidium bromide. BamHI linearises *CYP11B1* cDNA to generate a 6.3kb band. A single band of the correct size confirms the presence of the *CYP11B1* cDNA insert. Lane L contains 5µl HyperLadder I (Bioline, London, UK).

3.4.4 Confirmation of Mutations

The presence of each of the mutants (see **table 3.2**) was verified by sequencing using the automated sequencing method described in **section 2.2.6**. The entire insert was sequenced to rule out the incorporation of any unwanted mutations. Sequence data are shown in **figures 3.2a to h**. Sequencing revealed that the construct containing the L83S mutant had an extra unwanted mutation (see **figure 3.2b**) but, as this did not change the encoded amino acid, the construct was deemed suitable for use.

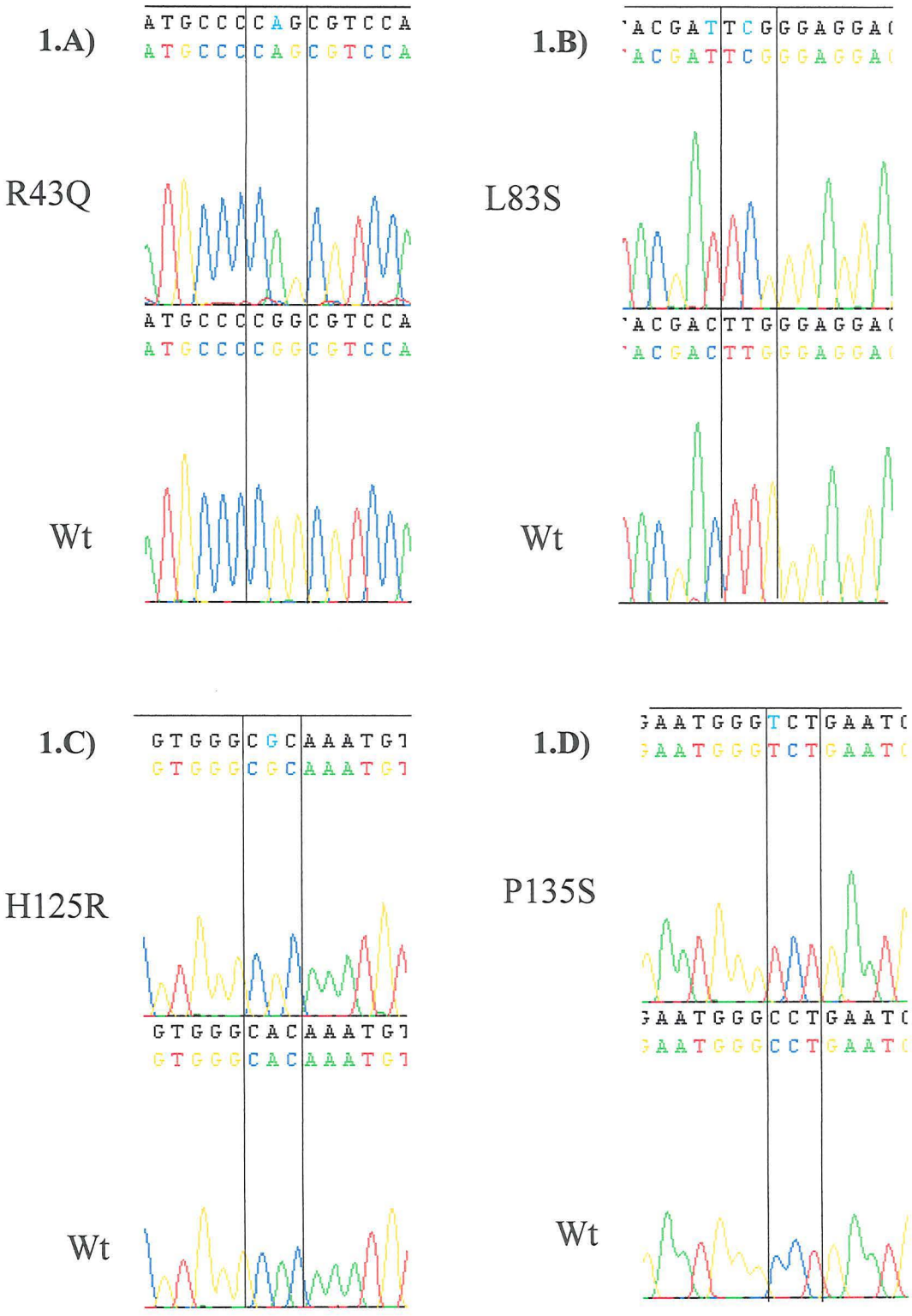


Figure 3.2 A to D. Sequences confirming presence of mutations R43Q, L83S, H125R and P135S respectively compared to wildtype pCMV4-B1.

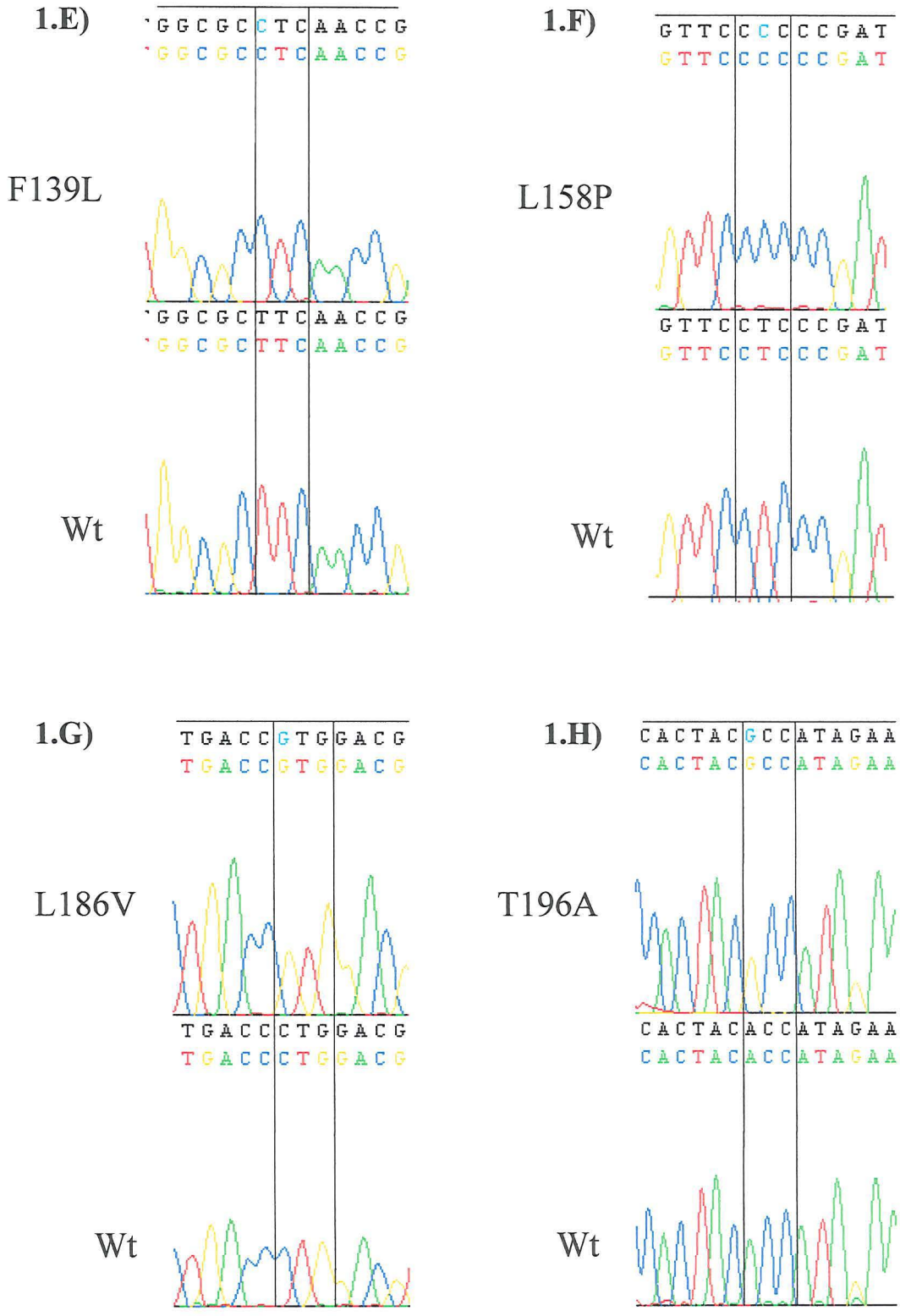


Figure 3.2 E to H. Sequences confirming presence of mutations F139L, L158P, L186V and T196A respectively compared to wildtype pCMV4-B1.

3.4.5 SF1 (-344 C/T) genotyping

The frequency of the -344 alleles was determined for each of the 4 populations, as shown in **table 3.3**. There was an overall 6% failure rate in the genotyping. The distribution of genotypes was as expected from previous studies (Davies *et al* 1999).

Group	CC	TC	TT	%C	%T
Normals	6 (0.18)	19 (0.57)	8 (0.24)	47%	53%
Unselected Hypertensives	8 (0.21)	23 (0.60)	7 (0.18)	52%	48%
ARR>750	6 (0.15)	22 (0.55)	12 (0.30)	43%	57%
ARR<200	8 (0.20)	17 (0.43)	15 (0.38)	41%	59%

Table 3.3 Genotype distribution for SF1 (-344 C/T) site

Chi-squared analysis revealed no significant difference in gene frequencies between groups ($X^2 = 4.59$, degrees of freedom = 6, P = 0.59).

3.4.6 Steroid Conversions

Graphs of the steroid products, cortisol (F) and corticosterone (B), against a range of substrate concentrations, 11-deoxycortisol (S) and 11-deoxycorticosterone (DOC) respectively, for the wildtype 11β-hydroxylase and with each of the mutants are shown in **figures 3.3 to 3.10**. All steroid results were corrected for transfection efficiency values. These were calculated by converting protein levels and luciferase activity of cell lysates to relative light units (RLU) luciferase per unit of protein. These values were then converted to fold differences of the mean wildtype control transfection. A comparison of the fold difference to wildtype was made for each of

the mutants at the lowest substrate concentration to give a rough idea of the potency of the effects.

3.4.6.1 Effects of Mutants over a range of substrate concentrations

The potency of the effects of the 8 mutations on 11β-hydroxylation of both substrates over a range of concentrations fell roughly into 3 groups (see **figures 3.3 to 3.10**).

Severe inactivation with regard to both substrates

Mutants L158P and L83S both severely reduced 11β-hydroxylase conversion of both substrates (S and DOC) at all concentrations tested (**figures 3.3a and b and 3.4a and b**). Their activities vary between absolutely no conversion at the lowest substrate concentration to a very small level of product observed (approximately 1 to 3% that of the wildtype) at the highest concentration of S and DOC tested.

Partial inactivation; similar response for both substrates

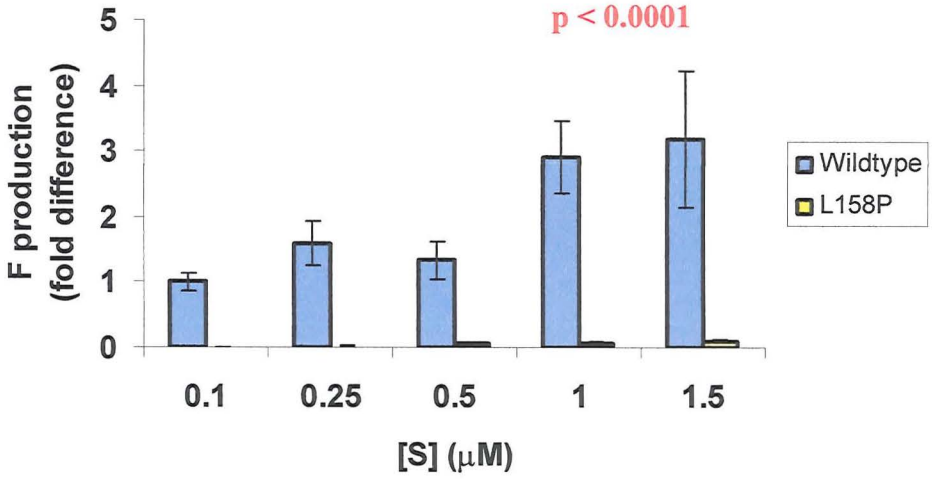
Mutant P135S greatly reduced the conversion of substrate S with product formation between approximately 2 to 10% that of the wildtype over the range of substrate concentrations (**figure 3.5a**). The effect on DOC conversion was less severe with product levels that varied between 10 to 40% that of the wildtype with increasing substrate concentration (**figure 3.5b**). The F139L (**figure 3.6a and b**), R43Q (**figure 3.7a and b**) and T196A (**figure 3.8a** and **b**), mutants resulted in product levels that were approximately 30% to 50% of the appropriate wildtype product with little variation in the potency of the effect over the range of substrate concentrations.

Markedly different effects for the two substrates

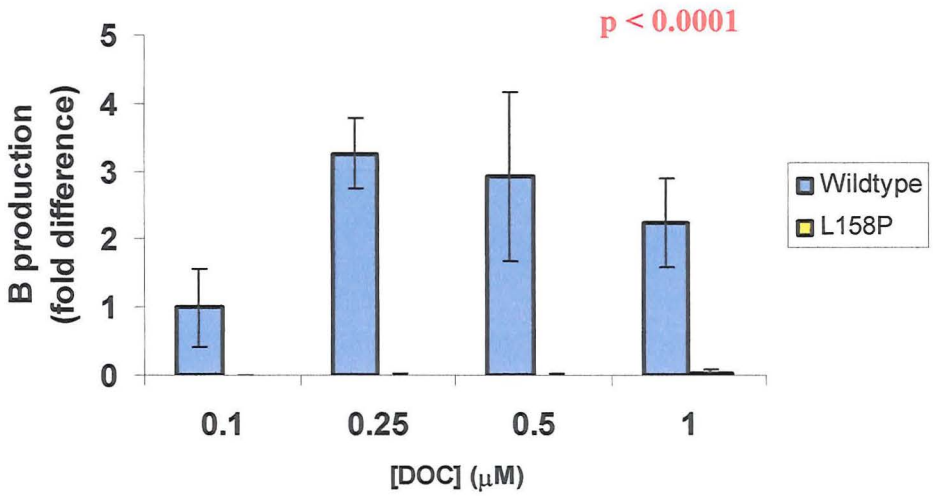
The H125R mutant had a substrate-specific effect, reducing conversion of S to F when compared to the wildtype (**figure 3.9a**) but not conversion of DOC to B (**figure 3.9b**). The extent of the reduction in the conversion of S to F was maintained over the range of concentrations tested. The mutant L186V also had a substrate specific effect; it increased the conversion of S to F over the range of concentrations tested compared to the wildtype (**figure 3.10a**) but did not significantly affect the conversion of DOC to B (**figure 3.10b**). The magnitude of the increase in product formation was maintained over the various concentrations of S. There appeared to be an increased product formation compared to wildtype at the lowest concentrations of DOC tested. However, over the range of concentrations tested this was not significant.

Figure 3.3 Effects of mutant L158P (Leucine-Proline)

A



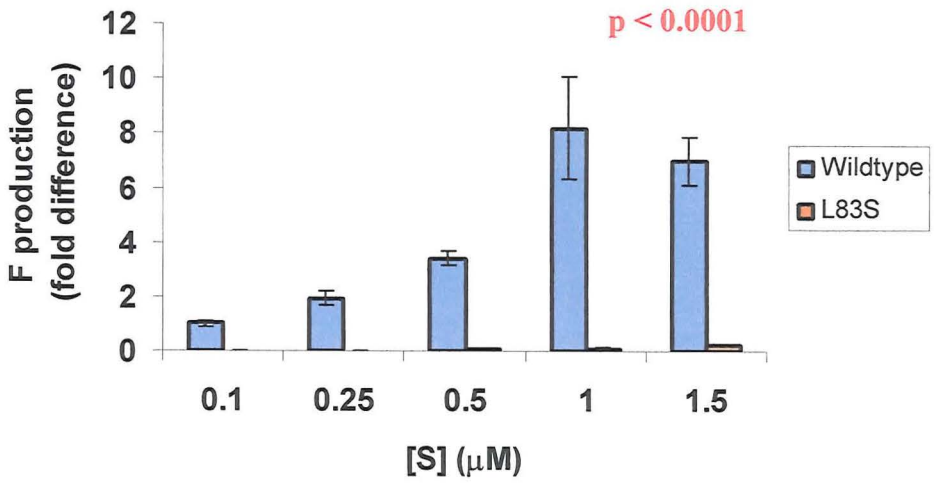
B



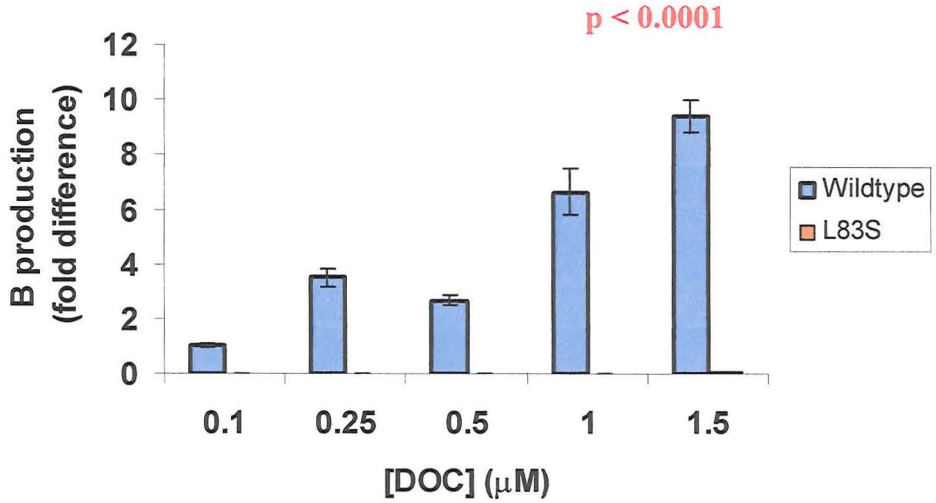
Effects of mutant L158P on the conversion of S to F (A) and DOC to B (B) compared to wild type pCMV4-B1. Results are expressed as a fold difference of the Wildtype product at 0.1μM substrate Values are the mean ± SEM (n = 6). A two-way ANOVA was used to compare the wildtype and mutant over the range of concentrations studied; the resulting p values are shown.

Figure 3.4 Effects of mutant L83S (Leucine-Serine)

A



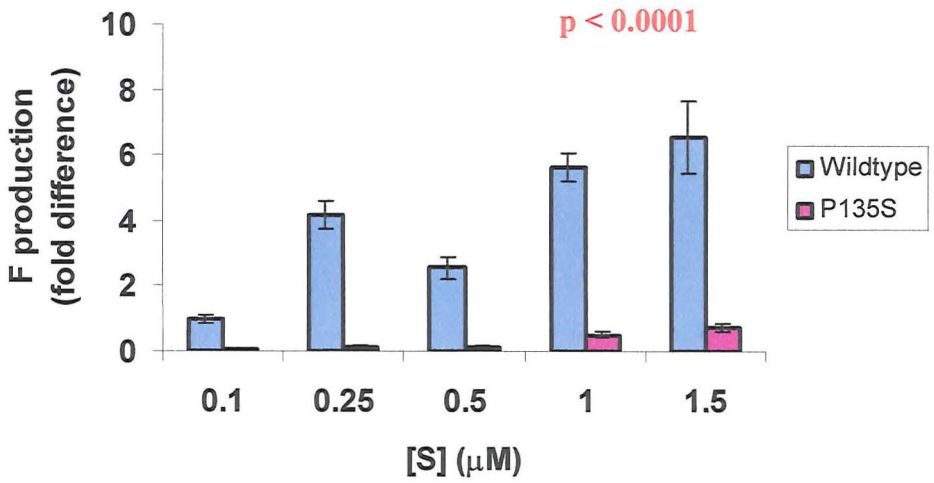
B



Effects of mutant L83S on the conversion of S to F (A) and DOC to B (B) compared to wild type pCMV4-B1. Results are expressed as a fold difference of the Wildtype product at 0.1μM substrate. Values are the mean ± SEM (n = 6). A two-way ANOVA was used to compare the wildtype and mutant over the range of concentrations studied; the resulting p values are shown.

Figure 3.5 Effects of mutant P135S (Proline-Serine)

A



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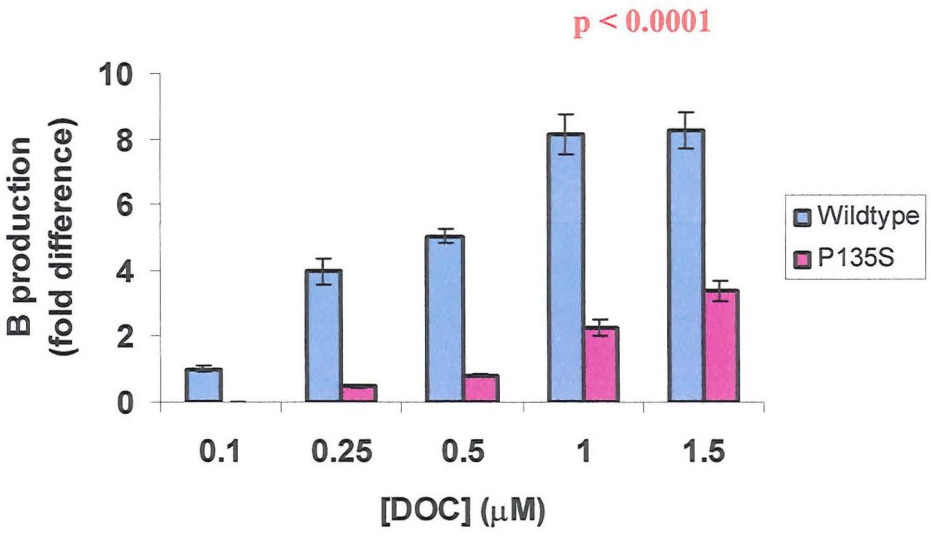
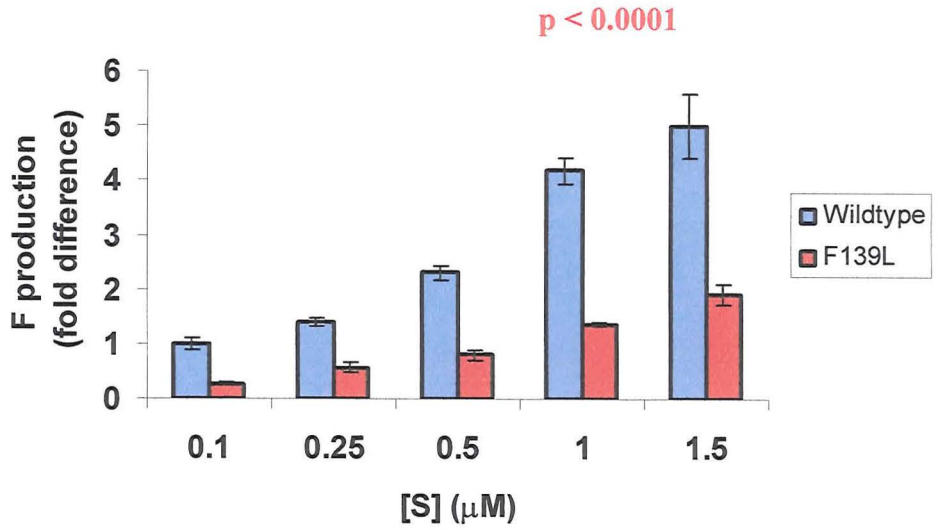


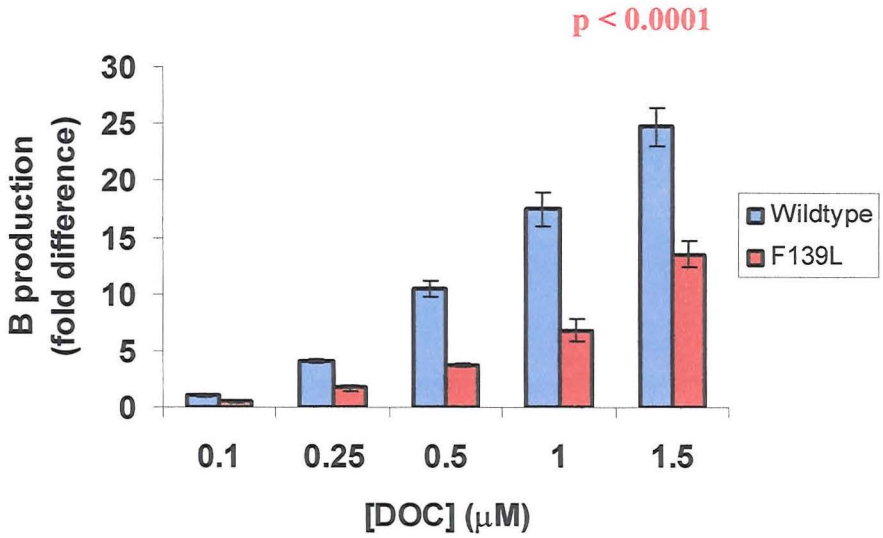
Effects of mutant P135S on the conversion of S to F (A) and DOC to B (B) compared to wild type pCMV4-B1. Results are expressed as a fold difference of the Wildtype product at 0.1μM substrate. Values are the mean ± SEM (n = 6). A two-way ANOVA was used to compare the wildtype and mutant over the range of concentrations studied; the resulting p values are shown.

Figure 3.6 Effects of mutant F139L (Phenylalanine-Leucine)

A



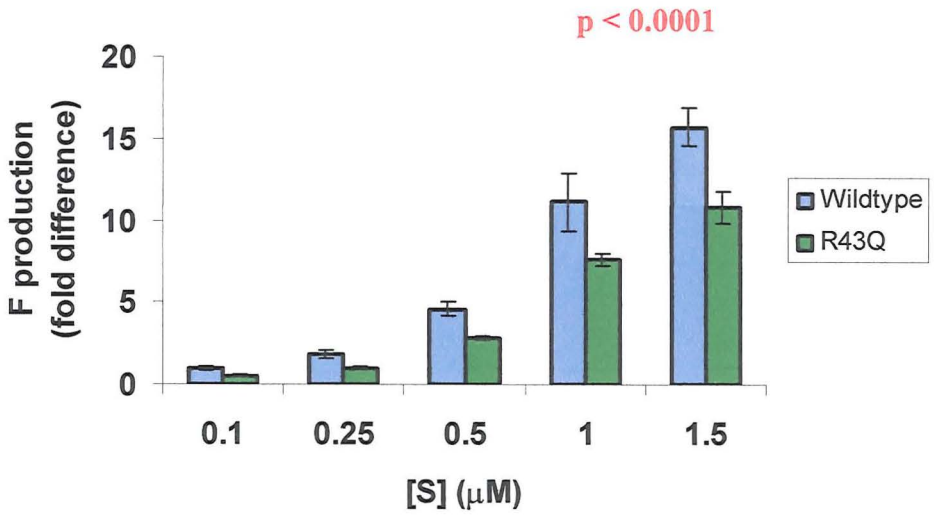
B



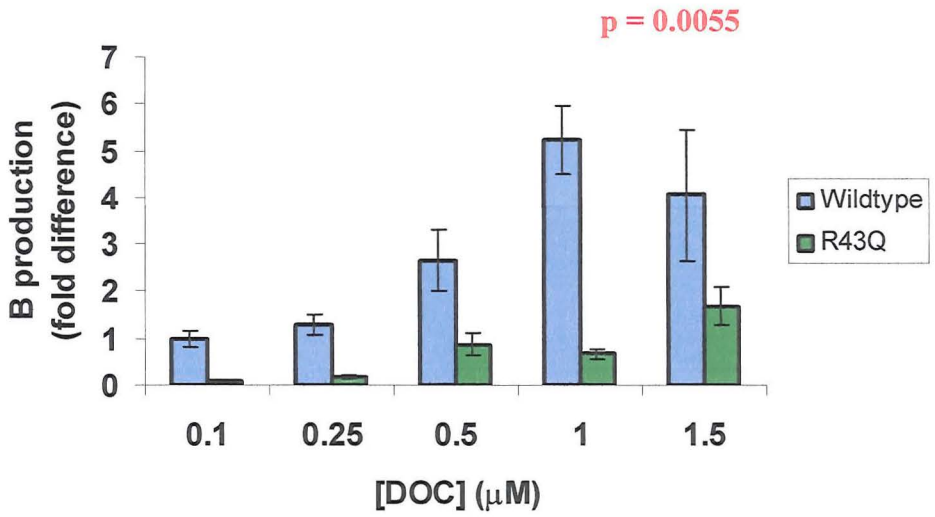
Effects of mutant F139L on the conversion of S to F (A) and DOC to B (B) compared to wild type pCMV4-B1. Results are expressed as a fold difference of the Wildtype product at 0.1μM substrate. Values are the mean ± SEM (n = 6). A two-way ANOVA was used to compare the wildtype and mutant over the range of concentrations studied; the resulting p values are shown.

Figure 3.7 Effects of mutant R43Q (Arginine-Glutamine)

A



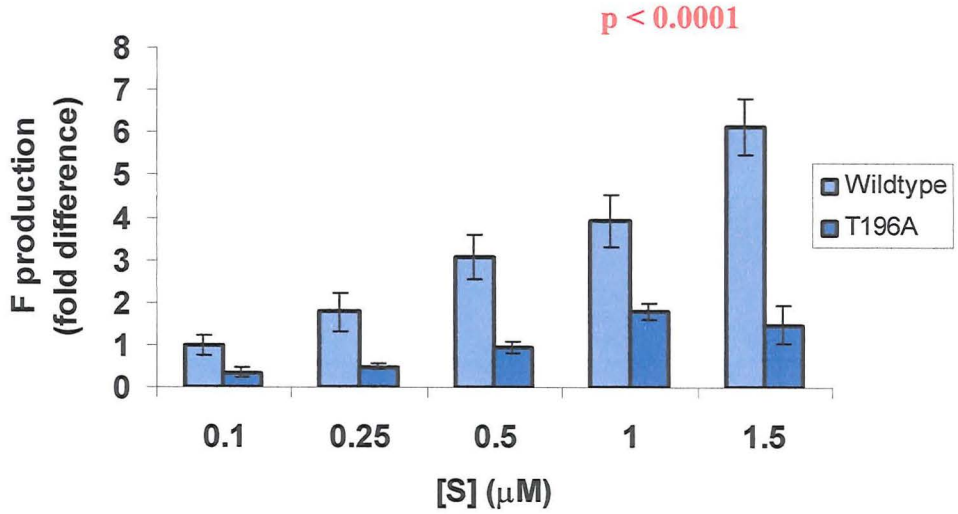
B



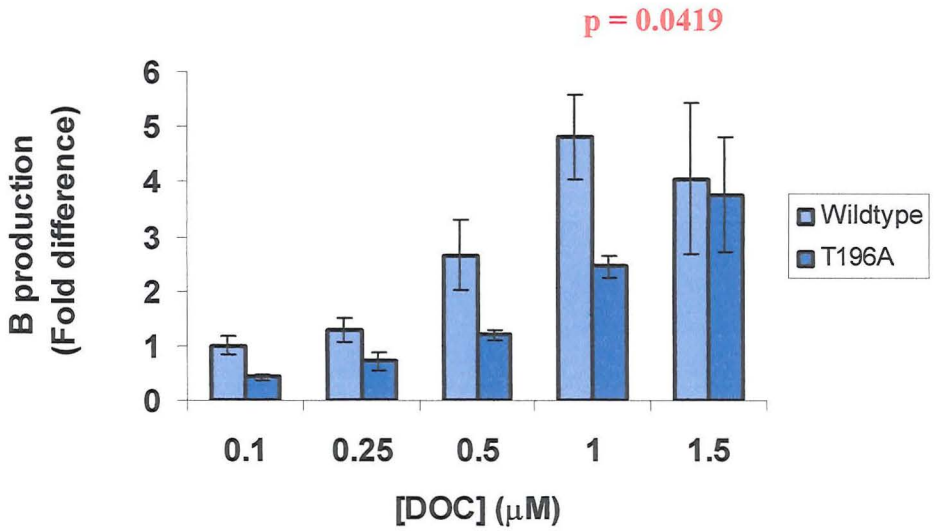
Effects of mutant R43Q on the conversion of S to F (A) and DOC to B (B) compared to wild type pCMV4-B1. Results are expressed as a fold difference of the Wildtype product at 0.1μM substrate. Values are the mean ± SEM (n = 6). A two-way ANOVA was used to compare the wildtype and mutant over the range of concentrations studied; the resulting p values are shown.

Figure 3.8 Effects of mutant T196A (Threonine-Alanine)

A



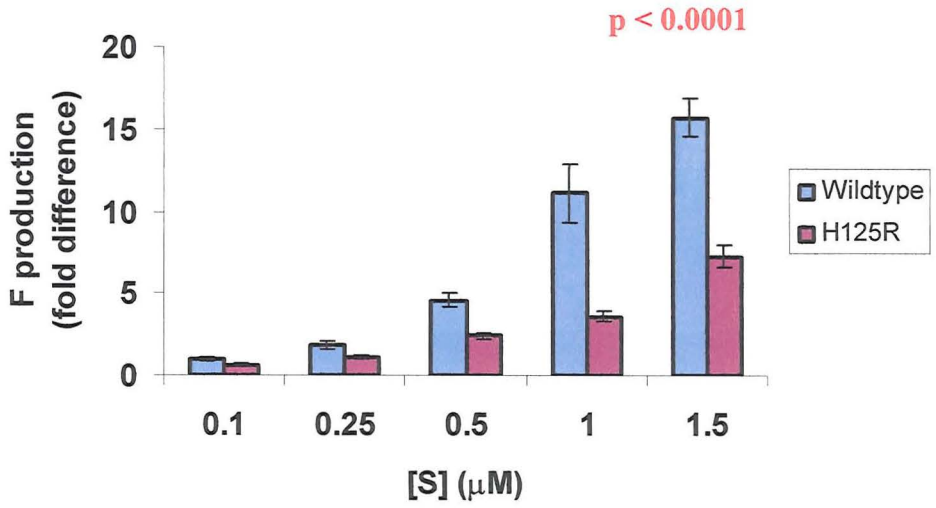
B



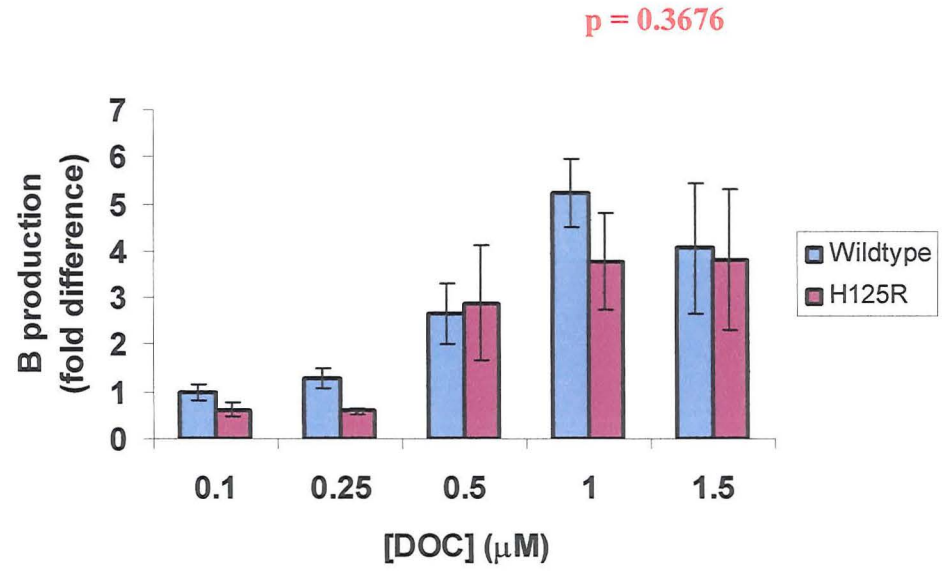
Effects of mutant T196A on the conversion of S to F (A) and DOC to B (B) compared to wild type pCMV4-B1. Results are expressed as a fold difference of the Wildtype product at 0.1μM substrate. Values are the mean ± SEM (n = 6). A two-way ANOVA was used to compare the wildtype and mutant over the range of concentrations studied; the resulting p values are shown.

Figure 3.9 Effects of mutant H125R (Histidine-Arginine)

A



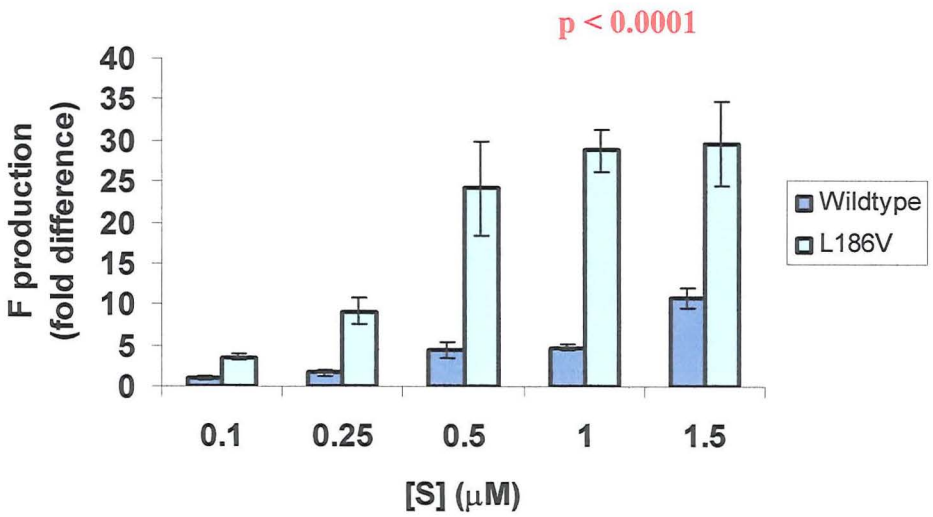
B



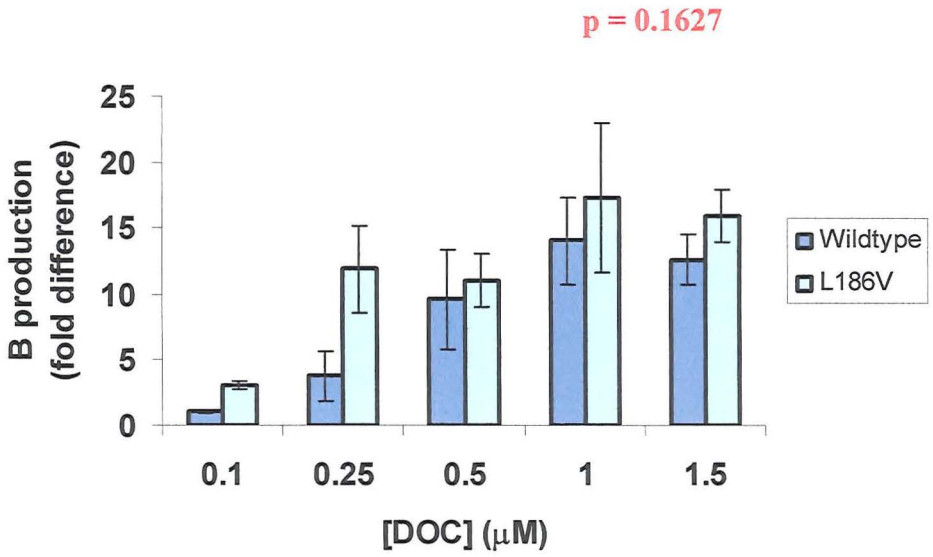
Effects of mutant H125R on the conversion of S to F (A) and DOC to B (B) compared to wild type pCMV4-B1. Results are expressed as a fold difference of the Wildtype product at 0.1μM substrate. Values are the mean ± SEM (n = 6). A two-way ANOVA was used to compare the wildtype and mutant over the range of concentrations studied; the resulting p values are shown.

Figure 3.10 Effects of mutant L186V (Leucine-Valine)

A



B



Effects of mutant H125R on the conversion of S to F (A) and DOC to B (B) compared to wild type pCMV4-B1. Results are expressed as a fold difference of the Wildtype product at 0.1μM substrate. Values are the mean ± SEM (n = 6). A two-way ANOVA was used to compare the wildtype and mutant over the range of concentrations studied; the resulting p values are shown.

3.4.6.2 Comparison of fold differences in product formation

Comparing the fold differences of each of the mutants to the wildtype at a single concentration allows a rough comparison of the severity of each of the mutants. This was done at the lowest concentration of substrate (0.1μM) which should maximise the distinction between differences in affinity (see **table 3.4**). Using this method, the potency of the effects of the 8 mutations on 11β-hydroxylation fell roughly into 4 groups:

Severe inactivation with regard to both substrates

The p values indicate that the mutants L83S, L158P and P135S have the most detrimental effects, with very poor conversion from S to F observed for L83S and P135S and no conversion of DOC. There was no conversion of either substrate for L158P.

Partial inactivation; similar response for both substrates

The mutants R43Q and F139L and T196A have less severe effects on enzyme function, resulting in partial reduction in conversion of both S and DOC.

Markedly different effects for the two substrates

The mutant H125R has a substrate specific effect with a significant inhibition of the conversion of S to F but not of DOC to B.

Increased activity with regard to both substrates

Mutant L186V has a unique effect, resulting in an increase in the conversion of both substrates.

Mutant	Mean F Production		Mean B Production	
	Fold diff to Wt ± SEM	**p value**	**Fold diff to Wt ± SEM**	**p value**
L158P	no activity	0.000	no activity	0.000
L83S	0.02 ± 0.01	0.000	no activity	0.000
P135S	0.06 ± 0.01	0.001	no activity	0.000
R43Q	0.53 ± 0.05	0.009	0.07 ± 0.01	0.002
F139L	0.27 ± 0.03	0.002	0.47 ± 0.04	0.003
T196A	0.35 ± 0.13	0.042	0.43 ± 0.06	0.018
H125R	0.63 ± 0.04	0.022	0.6 ± 0.06	NS
L186V	5.59 ± 2.09	0.002	3.07 ± 0.35	0.007

Table 3.4. Mean product levels as a fold difference of the wildtype product ± SEM for each of the mutants. Results for both the conversion of S to F and DOC to B are shown at 0.1μM substrate. The p values represent a comparison between the mutant and wildtype product levels using an unpaired 2-Sample t test.

NS = non-significant

In most cases, these observations are in agreement with the effects observed over the range of substrate concentrations. However, when comparing the range of concentrations using ANOVA, the mutant L186V only had a significant effect on the conversion of S to F and not on the conversion of DOC to B. In contrast, when comparisons are made at only the 0.1μM substrate concentration, there was a significant increase in the conversion of both substrates.

3.4.7 QRT-PCR

Real time QRT-PCR shows that the levels of *CYP11B1* transcript do not differ significantly between the wildtype plasmid and the mutant constructs studied (**figures 3.11 and 3.12**). Thus, the expression levels within the cells are likely to be similar and any observed changes in 11β-hydroxylase activity can be attributed to structural changes in the enzyme and not to changes in gene expression rate and subsequent protein production.

3.4.8 Modelling

A putative 3D Model of 11β-hydroxylase was generated using homology modelling techniques (see **figure 3.13**). From the model the mutant residues can be assigned to particular regions of the molecule:

Residue	Putative 3D location	Effect on 11β-hydroxylation
43	Rope/loop flanking helix A (exterior of molecule)	Partial reduction
83	Roop/loop flanking helix B	Severe reduction
125	Roop/loop between helices B and C	Substrate specific (↓ S to F)
135	Helix C	Partial reduction
139	Helix C	Partial reduction
158	Start of helix D	Severe reduction
186	Start of E' helix	Substrate specific (↑ S to F)
196	Start of helix E	Partial reduction

Table 3.5 Putative 3D locations of each of the mutant residues

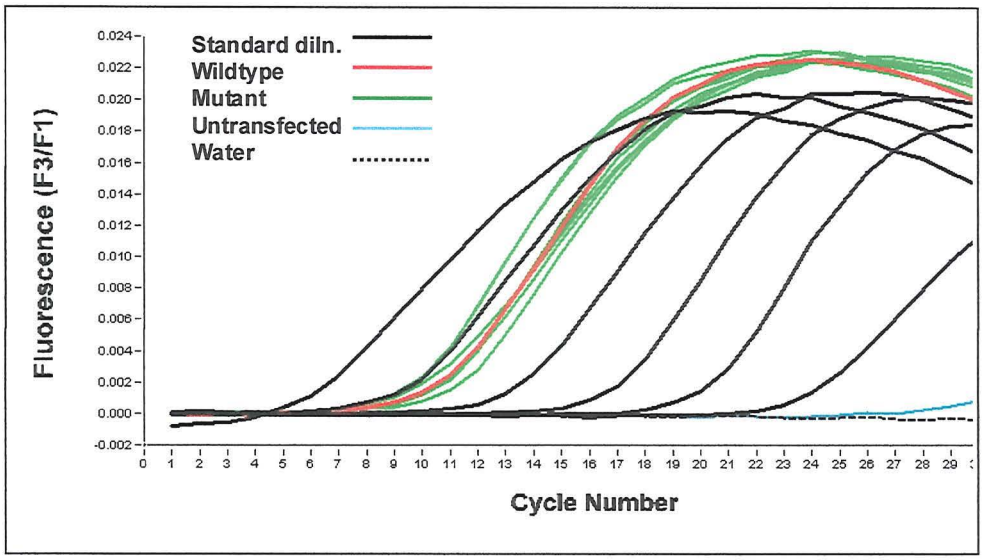


Figure 3.11 QRT-PCR Amplification Curve

Amplification curve displaying fluorescence values versus cycle number. Serial 10-fold dilutions of *CYP11B1* mRNA standard are shown in black. Sample copy number for the wildtype and each of the mutants was extrapolated from the standard curve on the base of the samples crossing point. This is a representative curve that was repeated 3 times. A negative control (water blank) and also RNA extracted from untransfected cells was included in each run.

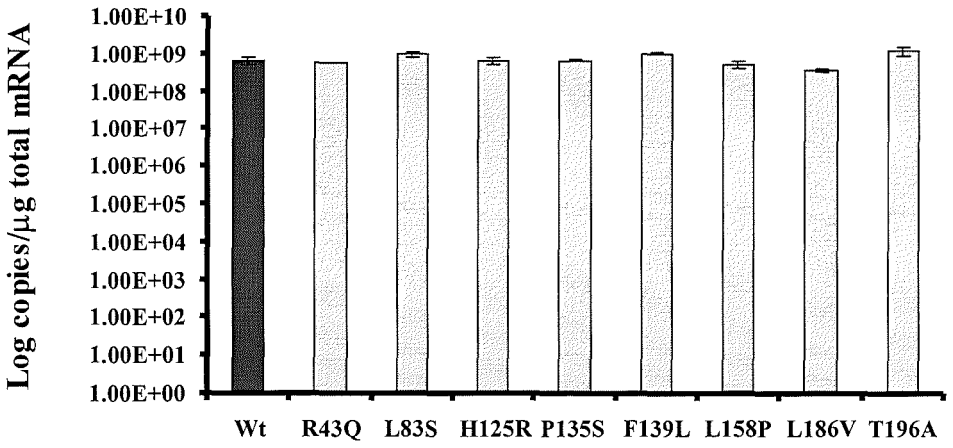


Figure 3.12 *CYP11B1* mRNA levels

Levels of *CYP11B1* mRNA from cells transfected with wildtype pCMV4-B1 and each of the mutant constructs. Values are expressed as mean mRNA copy number/mg total RNA ± SD from 3 independent experiments. Each of the mutants' mRNA copy number was compared to the wildtype using the nonparametric Mann Whitney test.

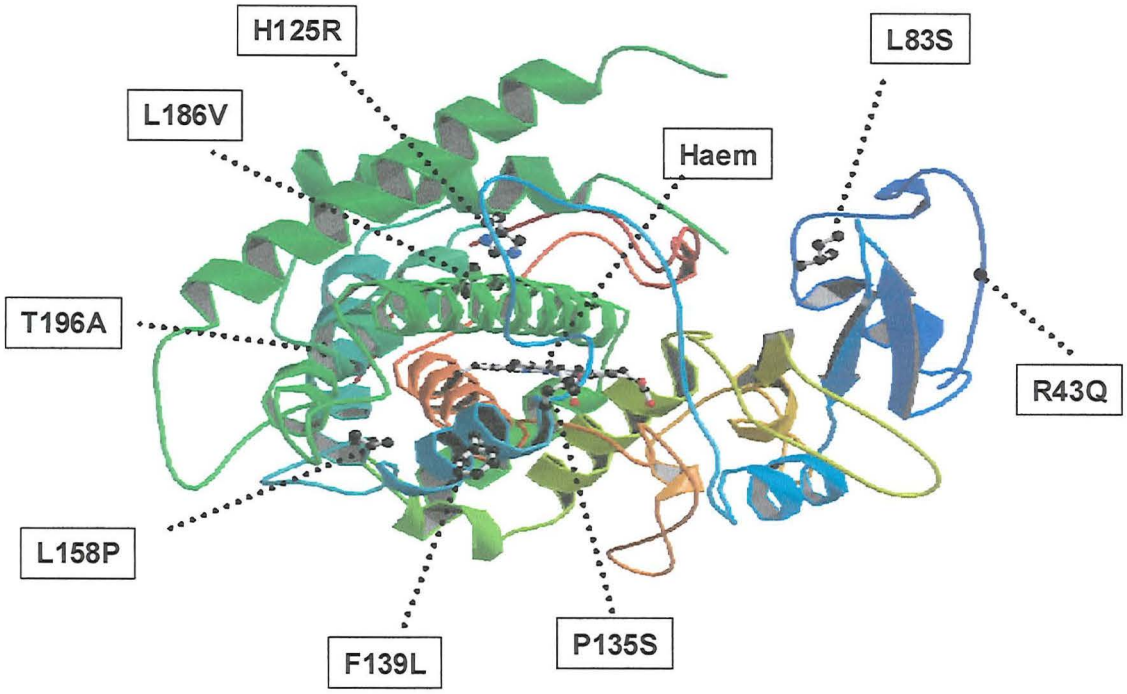


Figure 3.13 Homology Model of 11β-Hydroxylase

3-Dimensional model of the structure of human 11β-hydroxylase showing the positions of each of the mutant residues in relation to the active site containing the haem.

3.5 Discussion

These experiments investigated the presence of genetic polymorphisms in the *CYP11B1* gene. In particular they were concerned with whether such variation was associated with raised blood pressure and ARR and whether it affected the *CYP11B1*-dependent activity, 11β-hydroxylation. Several novel mutations were identified in the four groups of subjects, normotensive controls, unselected essential hypertensives or hypertensive subjects with either a high (≥ 750) or low ARR (≤ 200) that markedly change enzyme efficiency. However, their frequency was similar in normotensive and hypertensive groups and did not markedly differ when the low and high ARR groups were compared. It has previously been reported that the T variant of the -344 C/T polymorphism of the adjacent *CYP11B2* gene is found in higher frequency in hypertensive subjects, particularly in those with a raised ARR (Brand *et al* 1998; Davies *et al* 1999; Lim *et al* 2002b). This study was not powered to look for such an association; the study subjects were chosen on the basis of phenotype not genotype. The frequency of the T allele was very similar within the four groups (see **table 3.3**). It is therefore more likely that patients selected on the basis of genotype associated with a raised ARR might identify specific mutations that are linked to the development of this type of hypertension. In addition, this study examined only the coding region of *CYP11B1* and so it is possible that other mutations exist in the untranslated regions, either 5' or 3' of the gene, or located at exon/intron boundaries that may alter splicing, which associate with a raised ARR. These regions therefore require further investigation (see **Chapter 4**).

Although the variants identified did not appear to associate with hypertension or ARR, they had marked and varied effects on the activity of the resulting enzyme. It

could be argued that these changes in enzyme function are due to altered levels of expression of the modified proteins. However real time quantitative RT-PCR (QRT-PCR) was used to measure and compare *CYP11B1* transcript levels between the mutants and the wildtype. No significant differences were detected (**figures 3.11** and **3.12**). It would have been preferable to perform Western Blotting to demonstrate that protein expression was similar but, at the time of this study, a suitable antibody for 11β-hydroxylase was not readily available. An alternative approach would have been to use a construct containing a tag (eg. histidine tag) which would generate a labelled gene product. A commercially-available antibody raised against this label could then be used for Western Blotting. However the construct used in the present study was in a form that would have entailed a lot of extra cloning work and it was felt that the sensitivity of the RT-PCR approach made it the preferred method. Although the QRT-PCR technique does not give any indication of changes in expression levels, it confirms that the transcription of the mutant and wildtype cDNAs are similar, supporting the proposal that the observed changes in 11β-hydroxylase substrate conversion are due to structural changes in the protein. The effects of all the mutations will therefore be determined by the difference in the physical properties of the amino acids exchanged as well as the locus of the mutant residue within the protein.

The mutants L158P and L83S both severely reduced 11β-hydroxylase conversion of both substrates S and DOC over a range of concentrations (**figures 3.3** and **3.4**). Both mutations change the polarity of the encoded amino acid. The L158P mutant would also change the structure of the protein chain; the large cyclic proline residue would introduce a bend not present in the wildtype L158 form. A similar effect on the protein conformation would result from the P135S mutation, which again

decreased conversion of both substrates, although the effect on S conversion was much more pronounced than the effect on DOC conversion (**figure 3.5**). The mutant T196A also results in a change of polarity of the encoded amino acid. It has a more subtle effect than the previous mutants, resulting in product levels that are 30 to 40% compared to wildtype (**figure 3.8**). F139L and R43Q have a similar effect, reducing the level of product formation to between approximately 30 to 50% compared to the wildtype enzyme (**figures 3.6** and **3.7**). F139L is a fairly conservative change in amino acid, but R43Q might result in an altered charge. Arginine (R) often forms salt bridges through hydrogen bonding with negatively charged amino acids that are important for protein stability Disruption of this stability may account for the decreased enzyme function. The H125R mutation resulted in substrate-specific alterations in enzyme function, whereby conversion of S to F was decreased compared to the wildtype but DOC to B conversion was unaffected (**figure 3.9**). This suggests that a histidine at this position is important for the interaction with S. The mutant L186V had a unique effect that was also substrate-specific, increasing conversion of S to F but not that of DOC to B (**figure 3.10**). This is a fairly conservative amino acid change and how it alters the protein environment to favour increased S conversion is unclear.

In addition to the different physical properties of the amino acid at a given locus, the proximity of the locus to an important functional site will also alter enzyme efficiency and substrate specificity. More detailed understanding of the structure-function relationships of $P450_{11\beta}$ awaits elucidation of its 3-dimensional (3D) structure but some preliminary deductions can be made from the known structures of other cytochrome P450 enzymes. Studies of bacterial homologues suggest that P450 enzymes consist of a number of helices (A-L) (Ravichandran *et al* 1993) (see **section**

1.14). The haem in cytochrome P-450 enzymes is located between helices I and L. The P450 superfamily proteins have a conserved structural core (Presnell sr 1989) and so it is possible to use known crystalline P450 structures as templates for others. Homology modelling of $P450_{11\beta}$ suggests that the I helix (residues 301-331) contains many hydrophobic amino acids and is the putative active site (Belkina *et al* 2001). None of the mutations investigated in this study are located within this putative active site but they all clearly alter enzyme activity. It is possible that they disrupt one of the six proposed substrate recognition sites (SRS1-6) or that they alter the flexibility of the protein, affecting substrate access to the catalytic domain (Gotoh 1992).

Generating a 3D model of 11β-hydroxylase using the structure of the mammalian microsomal protein 21-hydroxylase (CYP2C5) as a template (Williams *et al* 2000) enabled these mutant residues to be assigned to particular regions of the molecule (see **figure 3.13** and **table 3.5**). From these putative 3D locations and results from previous structure-function studies, it is possible to speculate on the reason for some of these alterations in enzyme function.

Mutant R43Q

Residue Arg43 is located near the N terminus of the protein, which may be involved in protein-membrane interaction (Belkina *et al* 2001). Interestingly, a homozygous mutation at the adjacent locus (Pro42Ser) results in the non-classical form of 11β-hydroxylase deficiency with only partially reduced activity (see **section 1.15.2.3**) (Joehrer *et al* 1997). It seems likely that, if present as a homozygote, the mutation R43Q could produce a similar phenotype.

Mutant H125R

Residue 125 is located among a cluster of glycines (G123, G128 and G134) conserved in both 11β-hydroxylase and aldosterone synthase. These glycines are thought to be involved in the flexibility of the loop between helices B and C that contains the substrate recognition site 1 (SRS1) (see **section 1.14.3**) (Gotoh 1992; Krone *et al* 2005). Mutations within this region may alter substrate specificity and in fact mutant H125R appears to decrease the affinity for substrate S with no effect on DOC.

Mutants P135S and F139L

Residue 135 may form a bond with the haem moiety (see **figure 3.13**). Mutation at this locus, especially such a dramatic change as removal of the large orientation-changing cyclic proline residue (P135S), may alter the haem orientation and therefore the efficiency of electron transport in the oxidase reaction (see **section 1.7**). Residues 135 and 139 appear to be located within helix C, which is thought to form part of the substrate access channel. Again, mutations within this region have been asssociated with 11β-hydroxylase deficiency, both classical (Val129Met) and non classical (Asn133His) types (Geley *et al* 1996; Joehrer *et al* 1997).

Mutant L186V

Mutant L186V has a substrate specific effect but does not appear to be located within one of the proposed substrate recognition sites (see **section 1.14.3**) (Gotoh 1992). In the bacterial $P450_{CAM}$, residue 186 helps to maintain the substrate access channel in the closed conformation by binding to residue Asp251 in the I helix. Mutation of these residues favors substrate binding by allowing easier diffusion of the substrate

into the active site (Deprez *et al* 1994). Mutation at locus 186 (L186V) in human $P450_{11\beta}$ may also have a similar effect on the binding of S as it increases the conversion of S compared to wildtype. This is a substrate specific effect as the conversion of DOC is not affected (**figure 3.10**).

Mutations L83S , L158P and T196A

Mutations L158P, which is situated at the start of helix D, and L83S that lies in the loop just before helix B, severely reduce enzyme activity (**figures 3.1** and **3.2**). Residue 196 is located in helix E and also reduces enzyme activity but to a lesser extent (**figure 3.8**). From their putative positions and the information in the literature, the reasons for these effects are not entirely clear. Mutations at these sites may alter the position of the helix and change protein conformation. This may position the haem in an orientation less favourable for the 11β-hydroxylation of S or DOC. As mentioned previously, all these changes would result in a change in polarity and the L158P may also alter the geometry of the protein chain that might account for their detrimental effects on enzyme activity.

In summary, although these speculations are useful in partially accounting for the effects of sequence changes, a more complete explanation requires the determination of a high-resolution x-ray structure of $P450_{11\beta}$. None of the residues investigated are located within the putative active site of $P450_{11\beta}$ but do markedly alter enzyme function. Their location provides further clues to the structure-function relationships of cytochrome P450 enzymes. Fisher *et al* similarly showed that changes in residue 147 of $P450_{aldo}$ or $P450_{11\beta}$, also distant from the active site, reduced activity (Fisher *et al* 2000).

Cytochrome P450 enzymes are unlike most other enzymes. They can bind more than one substrate, making them an exception to the classic 'lock and key' principle of the specificity of enzyme function. $P450_{11\beta}$ is particularly varied in the range of substrates on which it may perform both 11β- and 18- (and possibly 19-) hydroxylation. The different effects of the mutations investigated in this study on S and DOC hydroxylation may give important insights into enzyme mechanisms. The substrates S and DOC differ by the presence and absence respectively of a 17α-hydroxy group, giving them different polarity and therefore affecting their interaction with the (hydrophobic, hydrophilic) amino acids of the substrate-binding site. The substrate-specific effect of the mutants H125R and L186V suggests that these residues play an important role in the interaction of the 17α-hydroxyl group of S with the enzyme.

Since the initial work was conducted for this study, techniques to perform high-throughput screening by direct sequencing have become available. Our own experience, and that of others examining the *CYP11B* locus in normotensive subjects, suggests that variation across the entire region is high (Cargill *et al* 1999; Halushka *et al* 1999). Each of the mutations identified was in subjects who were heterozygous, so that a normal *CYP11B1* allele was present. No subject had clinical symptoms of 11β-hydroxylase deficiency, although more detailed biochemical phenotyping was not performed. As yet no individual with this disorder has been identified as homozygous for these mutations. The mutations causing 11β-hydroxylase deficiency are distributed across the entire gene but with a slightly higher frequency in exons 2, 6, 7 and 8 (Curnow *et al* 1993; Geley *et al* 1996) (see **section 1.15.2.3**). The causative mutations can be insertions, deletions, nonsense or missense mutations

that usually result in a complete loss of enzymatic activity. A residual 11β-hydroxylase activity of 3% is insufficient to prevent the clinical symptoms of classic 11β-hydroxylase deficiency (Krone *et al* 2005).

A total of 8 mutations were identified in 160 subjects screened (and other studies have also identified a high frequency variation). The carrier rate for mutations within this gene that would be predicted to cause significant alteration of 11β-hydroxylation efficiency within the population may be in the order of 3 to 5%. This might be expected to give rise to a compound heterozygote rate of 1 in 2500 live births. However, the reported rate of severe hydroxylase efficiency in a Caucasian population is very much lower than this (White *et al* 1994). One possibility is that that severe mutations, such as L83S and L158P, are embryonically lethal so that the live birth rate would be substantially lower than this. However this seems unlikely as even complete loss of 11β-hydroxylase is not lethal *in utero* due to maternal cortisol crossing the placenta. Alternatively, it is possible that a substantial number of compound heterozygotes carry mutations that reduce but do not abolish 11β-hydroxylase activity, resulting in a form of non-classical 11β-hydroxylase deficiency. As mentioned previously mutations causing a nonclassical form of 11β-hydroxylase deficiency have been identified more recently, that cause partially disrupted enzyme activity *in vitro* to 15-37% of the wildtype (see **section 1.15.2.3**) (Joehrer *et al* 1997). In this circumstance, the phenotype is less severe, so that the classic presentation of virilising congenital adrenal hyperplasia in early life is not present. Less marked reduction in 11β-hydroxylase activity gives rise to a milder phenotype, possibly associated with the development of hirsutism and/or hypertension in later life. The mutations R43Q, P135S, F139L and T196A appear to

reduce 11β-hydroxylase substrate conversion to approximately 30 to 50% of the wildtype. It seems likely that, if these were present as homozygotes, they would also result in a nonclassical phenotype. Interestingly, the mutants P135S, F139L and T196A were all identified in a single subject. It is possible these and other mutations have not been identified due to the milder phenotype of this form of the disorder. The mutant H125R causes a mild reduction in the conversion of S to F with no effect on the conversion of DOC to B. How this would manifest itself *in vivo* is unclear.

The identification of as many as 5 mutants in 160 subjects that appear to partially reduce the conversion of substrate to product *in vitro* suggests that subtle forms of 11β-hydroxylase deficiency may be more common than has been generally perceived. Other studies have, indeed, reported that altered 11-hydroxylation efficiency is a common feature of patients with essential hypertension, although such studies have not carried out a systematic examination of the *CYP11B1* gene (Connell *et al* 1996; de Simone *et al* 1985; Honda *et al* 1977). Analysis of the polymorphic variation of a much larger hypertensive population, with more extensive phenotypic data will allow a better determination of the true frequency of the variance as well as in association between polymorphisms and phenotypes such as hypertension with mineralocorticoid features.

CHAPTER 4

Haplotype Analysis of the *CYP11B1/2* Locus

4.1 Introduction

As described in **section 1.16.3**, the -344 C/T and intron conversion (IC) variants in *CYP11B2* have been consistently associated with an impaired 11β-hydroxylase efficiency (Davies *et al* 2001; Hautanena *et al* 1998; Kennon *et al* 2004). It is likely that this seemingly paradoxical association can be explained by linkage disequilibrium (LD) between these *CYP11B2* variants and key polymorphisms in *CYP11B1* that could account for this phenotype. The -344 C/T and IC variants are indeed in linkage with polymorphisms in *CYP11B1*, specifically in exon 1 (225 G/A, Leu75Leu), intron 3 (2803 A/G) and intron 6 (4265 G/A) (Ganapathipillai *et al* 2005; Keavney *et al* 2005). The *CYP11B* haplotypes identified in each study associated with 11β-hydroxylase efficiency. However, the *CYP11B1* polymorphisms identified were either synonymous exonic or intronic and so are unlikely to account for the biochemical phenotype of impaired 11β-hydroxylase efficiency. Therefore, further fine mapping across the *CYP11B* locus is required to identify potential causative variants.

4.2 Aims

The aims of this study were to investigate the way in which genetic variation at the *CYP11B1/2* locus may lead to altered 11β-hydroxylase function through detailed analysis of the pattern of genetic variation across the whole locus. Through this it was also possible to determine the haplotype structure and extent of linkage disequilibrium across the entire *CYP11B* locus.

4.3 Methods

4.3.1 Samples

Genomic DNA was taken from a group of normotensive subjects already genotyped for the –344C/T and IC polymorphisms. Twenty six subjects were selected for genetic analysis; TT/ConCon (10), CC/WtWt (10) and TT/WtWT (6) (**sections 2.2.1** and **2.2.2**).

4.3.2 PCR and Sequencing

The *CYP11B1* gene from -2kb of the 5' untranslated region (UTR) to the end of exon 9 was amplified as a single PCR amplicon of approximately 7kb. The 3'UTR (750bp) was amplified separately (**section 2.2.3**). Genetic variants were identified by direct sequencing of the PCR products (**section 2.2.6**) in collaboration with other members of the group who sequenced the *CYP11B2* gene of the same subjects to determine the pattern of linkage across the entire *CYP11B* locus.

4.3.3 Haplotype analysis

A Bayesian statistical method implemented in PHASE v 2.1 (http://www.stat.washington.edu/stephens/software.html) was used to construct phased haplotypes from phase-unknown genotype data. Linkage disequilibrium (LD) statistics (D' and r^2) were generated using the programme EMLD (http://request.mdacc.tmc.edu/~qhuang/Software/pub.htm, which calculates LD between all possible pairs of genetic variants from unrelated individuals. This was further investigated using GOLD, which provides a graphical overview of LD (http://www.sph.umich.edu/csg/abecasis/GOLD/)

4.4 Results

4.4.1 PCR of 5'UTR to Exon 9 of CYP11B1

The 5′ UTR and coding region of *CYP11B1* was amplified using the oligonucleotides B1 5'UTR (s) and B1 exon 9 (as) to produce a fragment of approximately 7kb (see **figure 4.1**).

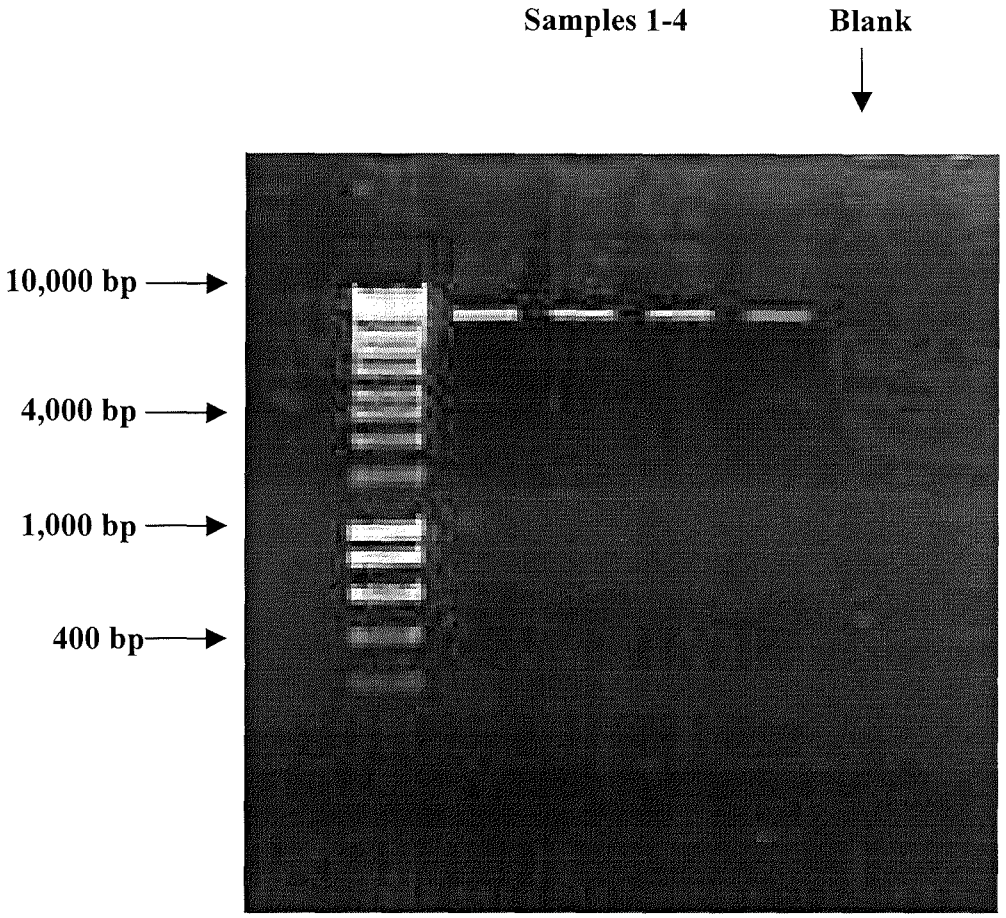


Figure 4.1. 7kb PCR fragments from 4 different MONICA samples resolved on a 1% agarose gel. Lane 1 contains 5µl HyperLadder I (Bioline, London, UK). Indicidual bands of the correct size indicate specificity. No band for the control/blank reaction indicates no contamination.

4.4.2 PCR of 3'UTR of CYP11B1

The 3'UTR of *CYP11B1* was amplified using the oligonucleotides M6635 and B13'UTR+500 (as) to produce a fragment of approximately 750bp (see **figure 4.2**).

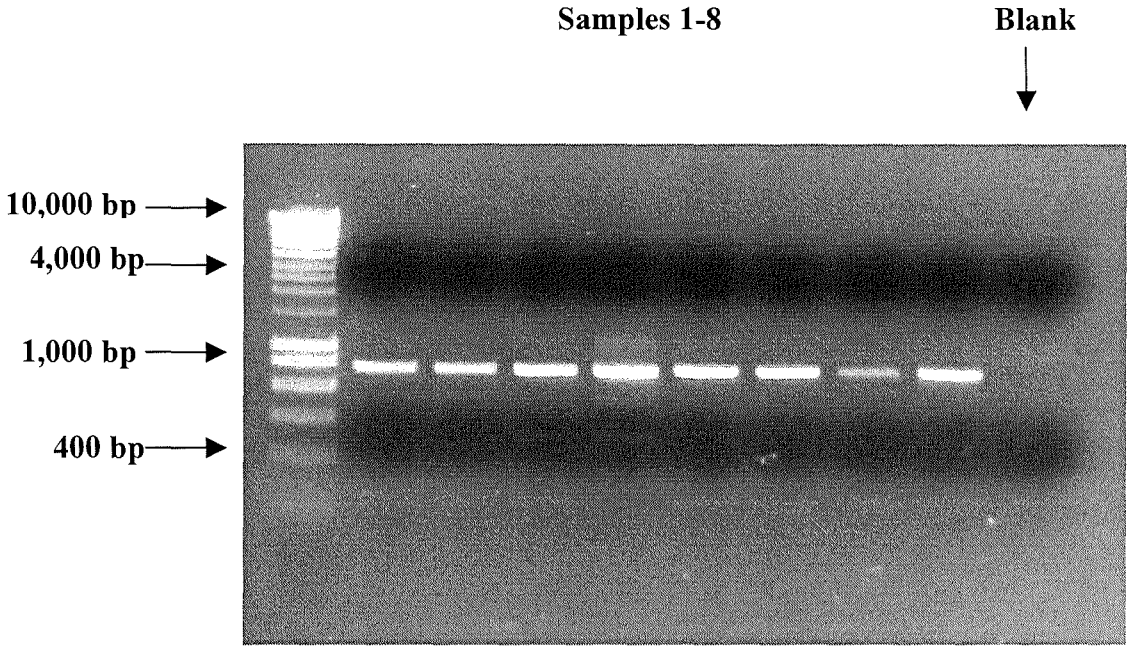


Figure 4.2. 750bp PCR fragments from 8 different MONICA samples resolved on a 1% agarose gel. Lane 1 contains 5µl HyperLadder I (Bioline, London, UK). Individual bands of the correct size indicate specificity. No band for the control/blank reaction indicates no contamination.

4.4.3 ***Polymorphism identification***

Polymorphisms were identified by direct sequencing of the genes. An example of electropherograms of the 5'UTR of *CYP11B1*, from subjects in each of the groups, are shown in **figure 4.3**. The sequence variation across the *CYP11B* locus was fairly high. For the purposes of this study, only the polymorphisms that differed between the three -344/IC genotype groups, in all subjects of the group, were chosen for further analysis (see **figure 4.4**). A total of 83 biallelic polymorphisms were identified across the entire *CYP11B* locus (44,631bp) that showed association with the -344 C/T and IC variants in *CYP11B2* (see **figure 4.5**). Out of the 83 polymorphisms, 64 were located within the *CYP11B2* gene and 19 were located within the *CYP11B1* gene. They were spread throughout the genes' exons, introns and their nearby untranslated regions (UTR), with the highest frequency in the intronic regions.

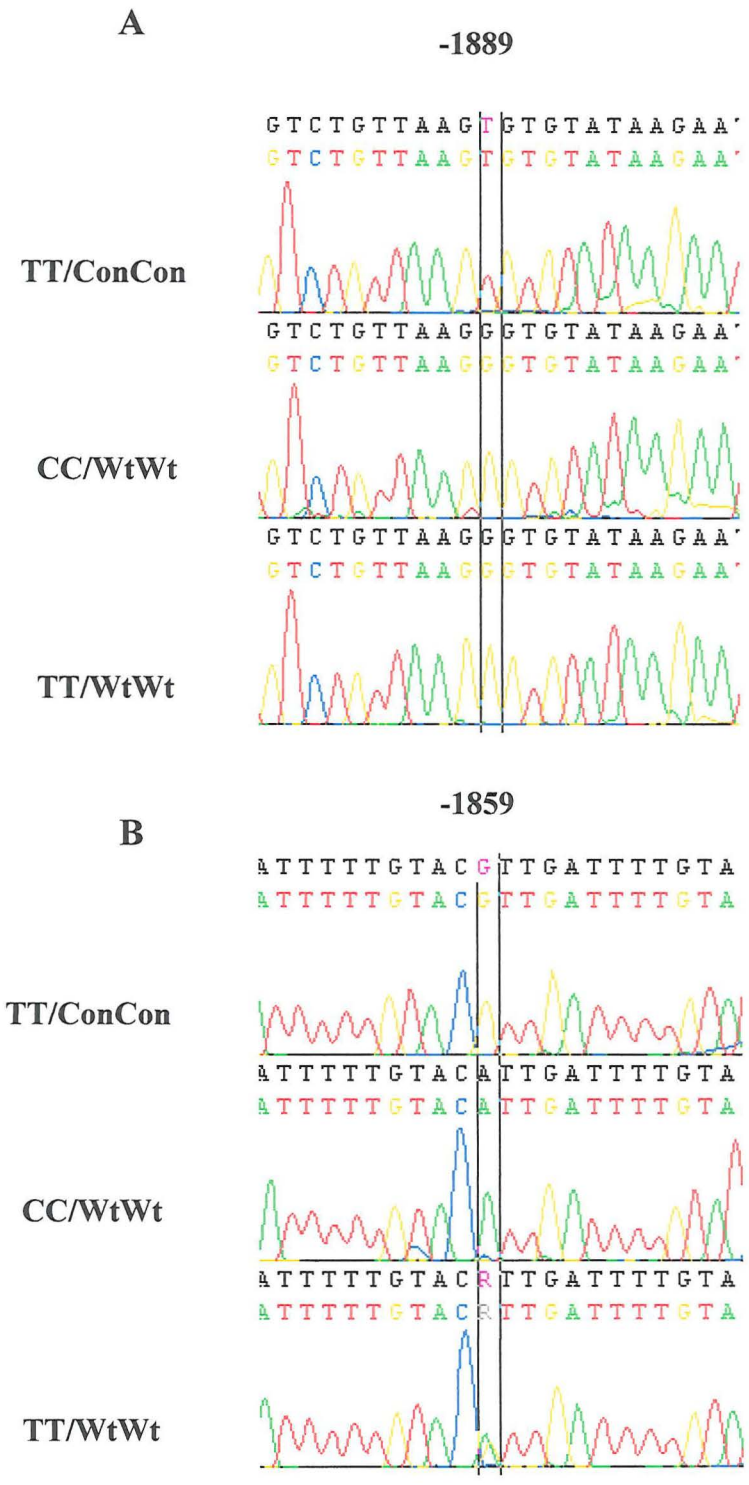


Figure 4.3. Sequence analysis showing two single nucleotide variants within the 5'UTR of *CYP11B1*. Results are shown from one subject from each of the 3 groups. A) G to T substitution at position -1889. B) A to G substitution at position -1859.

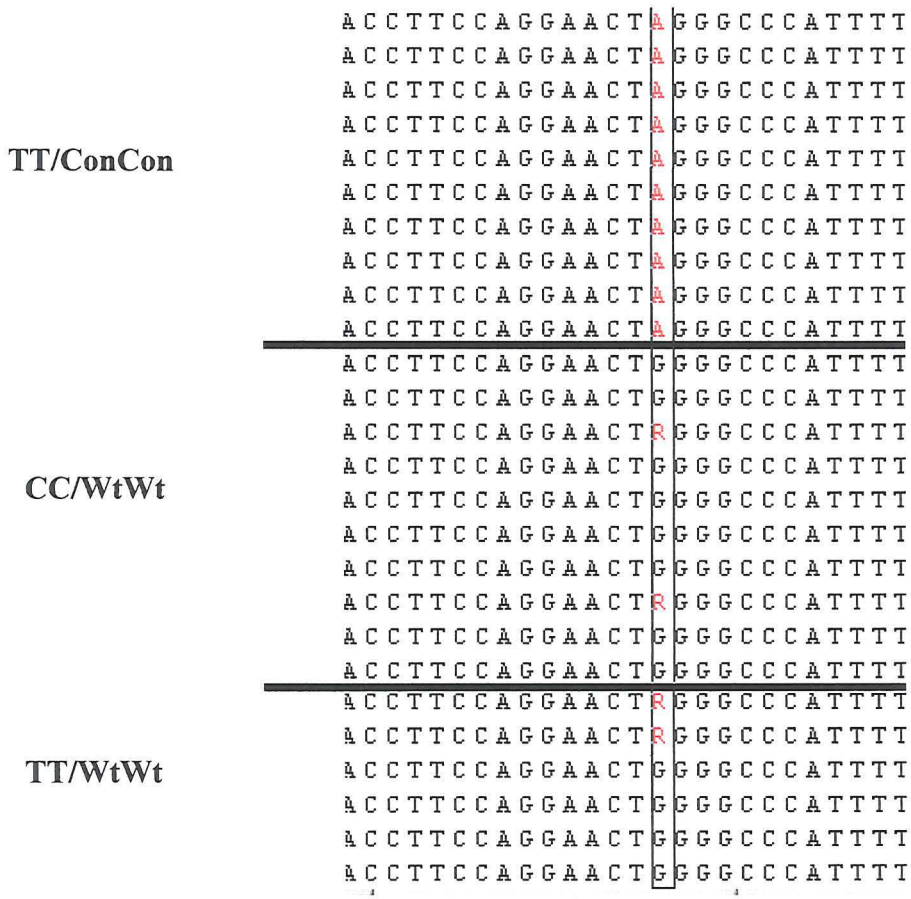


Figure 4.4. Sequence alignment of the three groups showing the presence of a CTG to CTA synonymous (Leu to Leu) polymorphism in codon 75 of exon 1, *CYP11B1*.

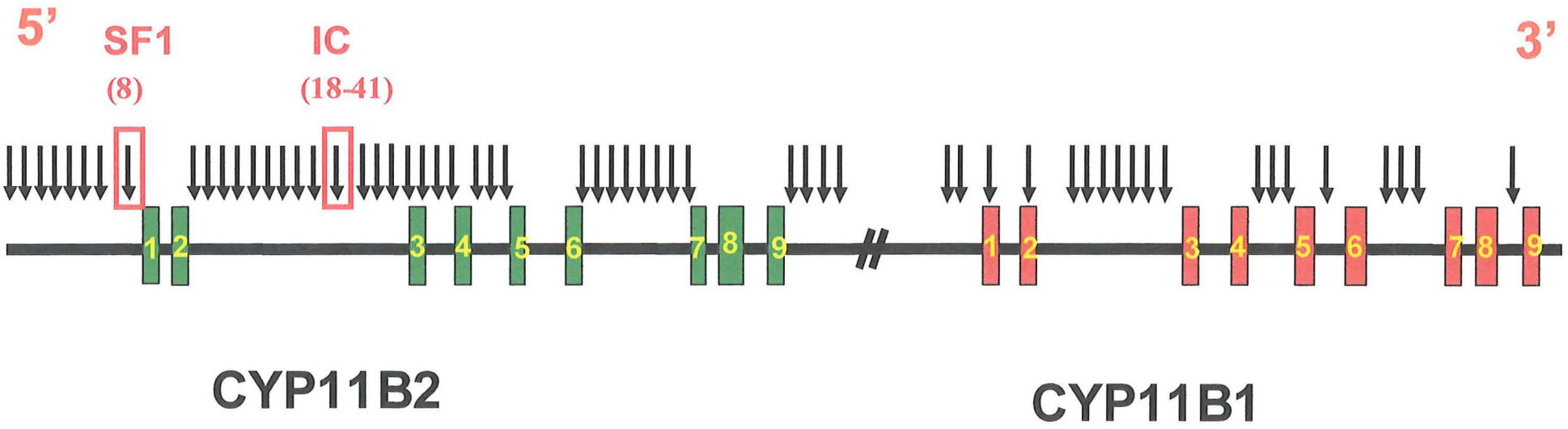


Figure 4.5. The positions of the 83 variations identified across the *CYP11B* locus. The SF1/-344C/T (No. 8) and the intron conversion (IC) (Nos. 18-41) variants are marked.

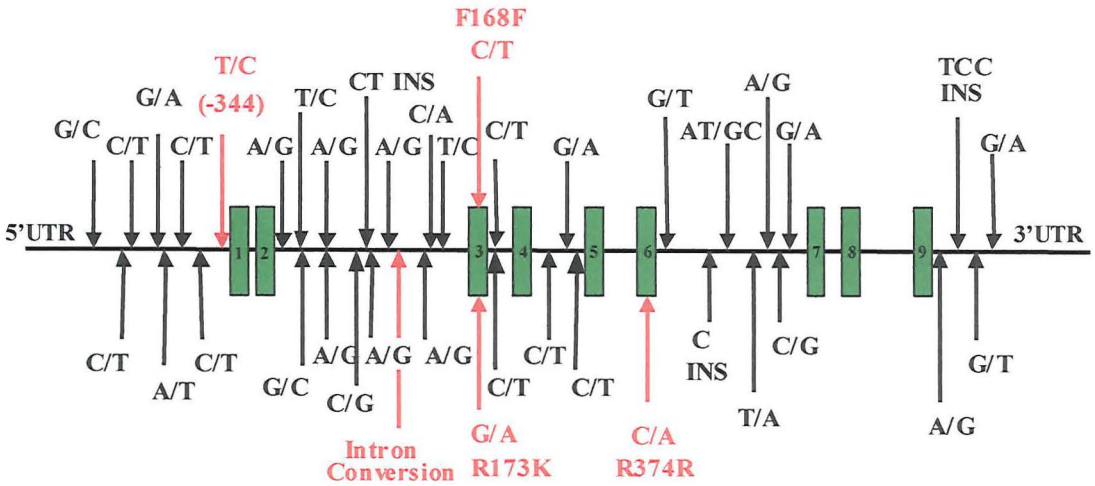
4.4.3.1 *CYP11B2* Polymorphisms

Sixty four polymorphisms were identified in *CYP11B2* that associate with the -344 C/T and IC polymorphisms (see **figure 4.6a**). Most of the polymorphisms identified were in intronic regions, particularly intron 2. The majority of these were associated with the intron conversion variant previously described by White & Slutsker, 1995, which is marked on the diagram (see **figure 4.6a and table 4.1**). This actually includes variants 18-42, spanning approximately 240bp, where the *CYP11B2* residue has been replaced by the corresponding *CYP11B1* residue. This includes an 8bp deletion (AGAGCTGC) that corresponds to the conversion allele allowing easy identification. Of the remainder, three single base substitutions were found in exons 3 and 6. Two of these are synonymous changes with no effect on amino acid sequence; Phe168Phe and Arg374Arg. These mutations have been previously described by the National Center for Biotechnology Information (NCBI), accessible on the dbSNP website (http://ncbi.nlm.nih.gov/SNP), with the identification numbers; rs 4546 and rs4538 respectively. The third coding variant, at codon 173 (rs4539), does cause an amino acid change from Lys to Arg but has been shown to have no effect on aldosterone synthase activity *in vitro* (Fardella *et al* 1996a; Portrat-Doyen *et al* 1998). Four variants were identified in the 3'UTR region. Finally, seven single base substitutions were identified in the 5'UTR region of *CYP11B2*, which can be linked to the -344/IC genotype. The pattern of association of these polymorphisms with the -344 C/T and IC variants is shown in **table 4.1.**

4.4.3.2 *CYP11B1* Polymorphisms

Nineteen polymorphisms were identified in *CYP11B1* that associate with the -344 C/T and IC polymorphisms in *CYP11B2* (see **figure 4.6b**). As with *CYP11B2*, most were within intronic regions (introns 2, 3, 4, 5, 6 and 8). Two of the single nucleotide substitutions in exons 1 and 2 have previously been described by NCBI. Both of these are synonymous changes; Leu75Leu and Asp82Asp (rs6410 and rs5283 respectively). The exon 1 polymorphism and two of the identified intronic variants (Int3 2776 A/G and Int 6 4224 T/C) (see **table 4.2**) are the polymorphisms previously found to associate with the -344 C/T and IC variants (Ganapathipillai *et al* 2005; Keavney *et al* 2005). Two novel polymorphisms were identified within the 5'UTR at positions -1889 (G/T) and -1859 (A/G) upstream of the transcription start site. When the polymorphisms are separated into alleles to look at the pattern of association with the -344/IC genotype, it can be seen that most of the polymorphisms associate with the T/Conv haplotype (as shown in red on **table 4.2**).

A) CYP11B2 Identified Polymorphisms



B) CYP11B1 Identified Polymorphisms

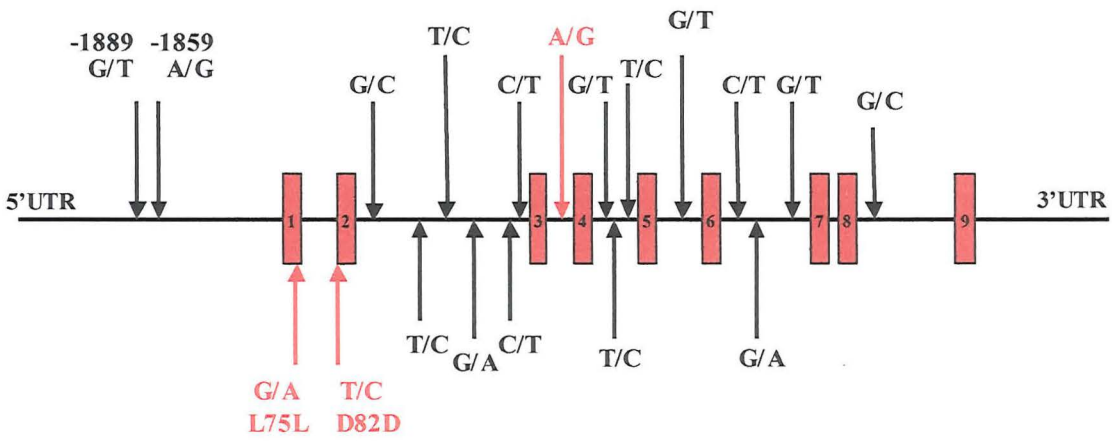


Figure 4.6. Diagram showing the positions of the identified polymorphisms and the nucleotide variants in a) *CYP11B2* and b) *CYP11B1*. Previously reported polymorphisms are shown in red.

***CYP11B2* alleles**

Location	5'	5'	5'	5'	5'	5'	5'	5'	Int2	Int2
Position	-1667	-1651	-1513	-1472	-663	-645	-470	-344	830	1049
TT/ConCon	C	C	C	G	A	T	C	T	A	T
CC/WtWt	G	T	T	A	T	C	T	C	G	C
TT/WtWt	G	C/T	C	G	A	C	C	T	G	C

Location	Int2	Int2	Int2	Int2	Int2	Int2	Int2	Int2	Int2	Int2
Position	1097	1461	1746	1750	1751	1782	1839	2095	2096	2100
TT/ConCon	G	A	G	C	-	A	A	G	C	C
CC/WtWt	C	G	A	G	CT	G	G	A	T	T
TT/WtWt	G	G	G	C	-	A	A	A	T	T

Location	Int2	Int2	Int2	Int2	Int2	Int2	Int2	Int2	Int2	Int2
Position	2101	2108	2110	2112	2114	2115	2117	2120	2122	2124
TT/ConCon	A	T	G	G	T	-	△8bp	-	-	-
CC/WtWt	G	A	A	A	A	G	-	A	T	C
TT/WtWt	G	A	A	A	A	G	-	A	T	C

Location	Int2	Int2	Int2	Int2	Int2	Int2	Int2	Int2	Int2	Int2
Position	2151	2240	2241	2252	2259	2264	2268	2269	2271	2287
TT/ConCon	A	G	T	C	T	C	C	C	-	C
CC/WtWt	G	C	C	G	G	A	T	G	T	T
TT/WtWt	G	C	C	G	G	A	T	G	T	T

Location	Int2	Int2	Int2	Int2	Int2	Ex3	Ex3	Int3	Int3	Int4
Position	2316	2328	2374	2375	2399	2698	2712	2886	2888	3192
TT/ConCon	G	G	C	A	T	C	A	C	T	T
CC/WtWt	T	A	A	G	C	T	G	T	C	C
TT/WtWt	T	A	C	A/G	T/C	C	A	C	T	T/C

Location	Int4	Int4	Ex6	Int6	Int6	Int6	Int6	Int6	Int6	Int6
Position	3257	3270	6573	6603	6855	6876	6879	6885	6887	6954
TT/ConCon	A	T	C	C	T	AT	T	C	C	A
CC/WtWt	G	C	A	A	T	GC	A	G	G	G
TT/WtWt	A/G	C	A	A	G	AT	T	C	C	G

Location	3'	3'	3'	3'
Position	7615	7829	8066	8269
TT/ConCon	A	-	G	G
CC/WtWt	G	TCC	T	A
TT/WtWt	G	TCC	T	G

Table 4.1. The identified *CYP11B2* polymorphisms separated into alleles to show the pattern of linkage with the -344/IC genotypes. The positions are relative to the transcription start site of *CYP11B2*. The -344 C/T and the IC (8bp deletion) variants are highlighted in light blue. The alleles that associate with the T/Con haplotype are shown in red and those that associate with the -344 C allele are shown in blue. For some polymorphisms the association was not clear, as subjects in the TT/WtWt group were heterozygous.

***CYP11B1* Alleles**

Location	5'	5'	Ex1	Ex2	Int2	Int2	Int2	Int2	Int2	Int2
Position	-1889	-1859	225	626	1052	1655	1800	1960	2265	2295
TT/ConCon	T	G	A	C	C	C	C	A	T	T
CC/WtWt	G	A	G	T	G	T	T	G	C	C
TT/WtWt	G	A	G	C	C	T	T	G	T	C

Location	Int3	Int4	Int4	Int4	Int5	Int6	Int6	Int6	Int8
Position	2776	3223	3244	3342	3799	4224	4264	4381	4941
TT/ConCon	G	T	C	C	T	T	A	T	C
CC/WtWt	A	G	T	T	G	C	G	G	G
TT/WtWt	A	G	T	T	G	C	G	G	G

Table 4.2. The identified *CYP11B1* polymorphisms separated into alleles to show the pattern of linkage with the -344/IC genotypes. The positions are relative to the transcription start site of *CYP11B1*. The alleles that associate with the T/Con haplotype are shown in red and those that associate with the -344 C allele are shown in blue.

4.4.4 Linkage disequilibrium

There was strong LD across the entire *CYP11B* locus (see **figure 4.7**). The LD decreased slightly with distance, with loci in *CYP11B1* being in stronger LD with each other than with loci in *CYP11B2* and vice versa. The D' value was almost uniformly high across the entire locus, approaching the maximum of 1.0 across much of the region and decreasing to approximately 0.75 between pairs of loci spanning *CYP11B1* and *CYP11B2*. The r^2 value was slightly lower as it is more stringent and takes into account allele frequency. However, it was still within the top part of the range with a minimum value of approximately 0.5 and a maximum of approximately 1.0.

4.4.5 Haplotype analysis

The theoretical number of haplotypes, assuming free recombination between the 83 variants, is 9.671×10^{24}. Haplotype analysis using PHASE identified 136 haplotypes that were conserved across the entire region; most of which were of a very low frequency. Nine haplotypes had fequencies > 1%, describing 75% of chromosomes in the data set (see **figure 4.8**). Only four common haplotypes had frequencies of greater than 5% and accounted for 68% of chromosomes in the data set (see **figure 4.9**).

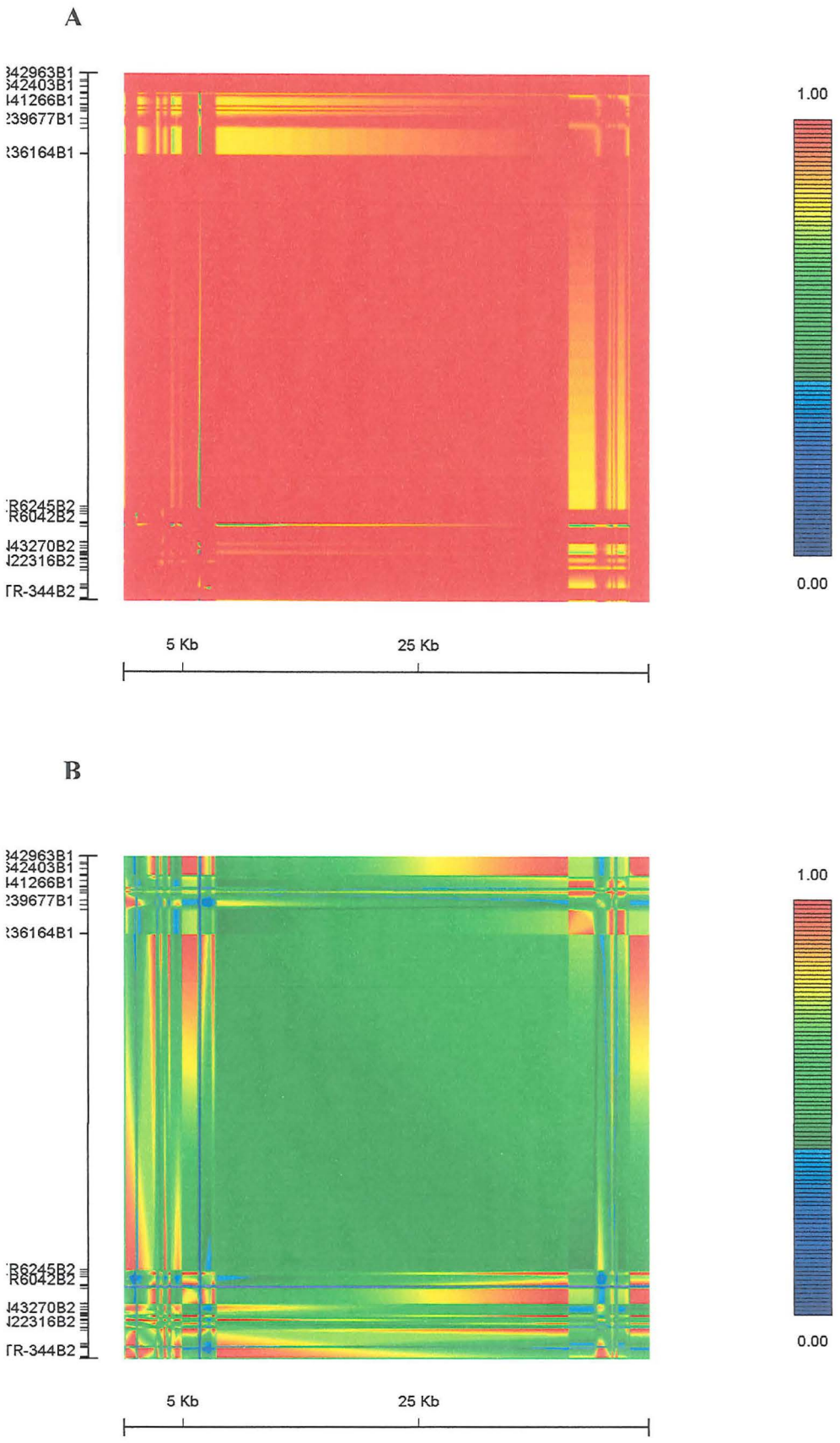


Figure 4.7. Data plots extrapolating pairwise linkage disequilibrium across the *CYP11B* region. A. Plot of D'. B. Plot of r^2.

No	Haplotype Pattern					Freq	%
1	1112121	2	122121111211122221	2	1111221221121122121112212221121111112221111112222122121	0.202	> 5
5	1112121	2	122121111211122221	2	1111221221121122121112212221121111112221111112222122121	0.014	> 1
9	1112121	2	122121111211122221	2	1111221221121122121112212221121111212221111112222122121	0.069	> 5
29	1112111	2	122121111211122221	2	1111221221121122121112212221121111112221111112222122121	0.112	> 5
87	2122111	2	212221111122211112	1	2222112112211222121112221111121122222112112122111211212	0.015	> 1
89	2122111	2	212221111122211112	1	2222112112211222121112221111121122222112112222111211212	0.011	> 1
132	2221212	1	211212222122211112	1	2222112112212211212221121122212222221121111112221121212	0.012	> 1
133	2221212	1	211212222122211112	1	2222112112212211212221121122212222221121211112221121212	0.022	> 1
136	2221212	1	211212222122211112	1	2222112112212211212221121122212222221112222221111221212	0.297	> 5

Figure 4.8. The 9 most common *CYP11B* haplotypes and their frequencies. Alleles coded 1 or 2 in alphabetical order. In the case of insertions/deletions, no insertion/deletion is 1; insertion/deletion is 2. The positions of the -344C/T and Intron conversion (8bp deletion) are shown. The 8bp deletion (2) is representative of the conversion allele whereas no deletion (1) is the wild type.

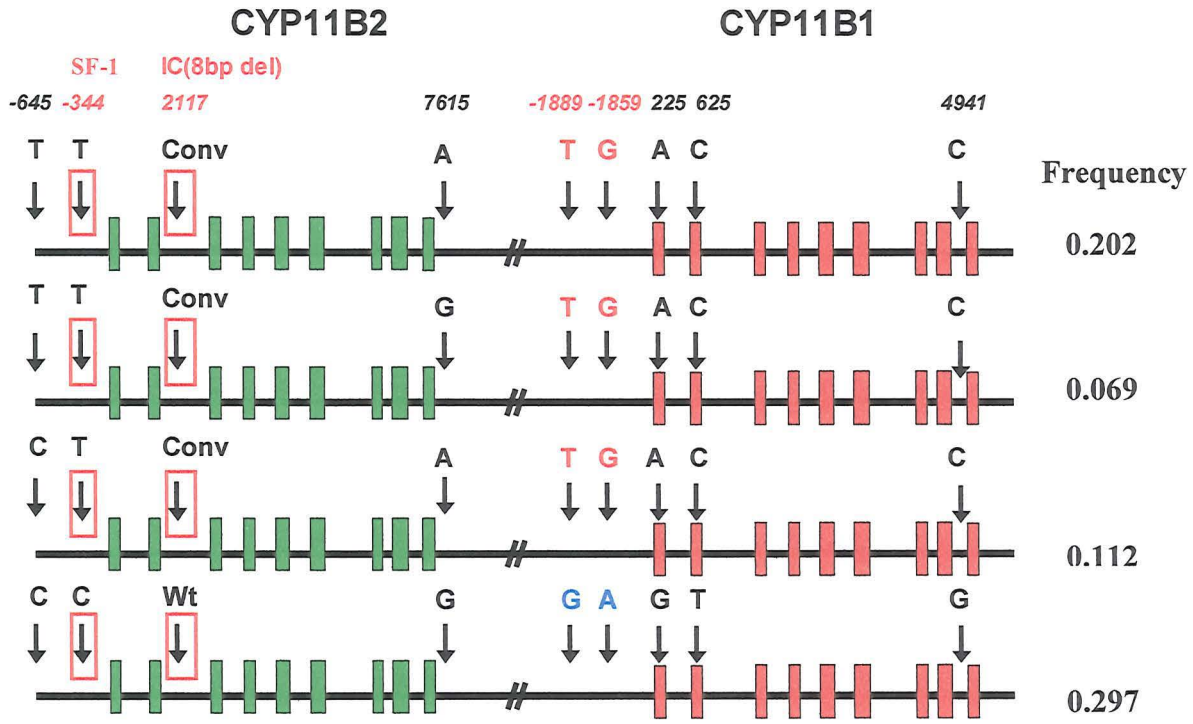


Figure 4.9. Schematic diagram of the 4 common haplotypes with a frequency of > 5%. The association of the SF1/IC variants with the -1889 G/T and -1859 A/G polymorphisms is highlighted. The *CYP11B2* T/Wt allele combination was only present in the haplotypes with a frequency of > 1%.

4.5 Discussion

This study demonstrates that overall the sequence variation across the *CYP11B* locus is high, supporting previous reports (Cargill *et al* 1999; Halushka *et al* 1999). Recent work has also suggested that the -344C/T and IC polymorphisms in *CYP11B2* are in linkage with specific polymorphisms in *CYP11B1*, in exon 1 (Leu75Leu), intron 3 (A/G) and intron 6 (G/A) (Ganapathipillai *et al* 2005; Keavney *et al* 2005). The present study also confirms this and further shows that there is tight linkage disequilibrium across the entire *CYP11B* locus. Several candidate SNPs have been identified that could account for the observed phenotypes associated with the -344C/T and IC variants in *CYP11B2*.

Out of the 64 identified polymorphisms in *CYP11B2* found to be in linkage with the -344/IC genotype, the only substitutions within the coding regions were either synonymous (F168F and R374R) or have previously been shown to have no effect on aldosterone synthesis (R173K) (Fardella *et al* 1996b). However, there are many polymorphisms located within introns. These could potentially alter the splicing of the gene's primary transcript leading to altered protein synthesis. Although none are located directly within the splice junctions at the exon/intron boundaries they may disrupt the branch site that is also necessary for proper intronic splicing. These are splicing-related sites located deeper within the intron with the consensus sequence (C/U)N(C/U)U(A/G)**A**(C/U) where the emboldened A (adenosine residue) is invariant and conserved between all genes and N is any nucleotide (Madhani & Guthrie 1994). To determine whether any of the intronic polymorphisms have functional effects on splicing could be studied using a minigene system where the entire exonic and intronic regions are cloned into a vector. The wildtype and mutant

constructs would then be transfected into JEG-3 cells, the total RNA isolated and reverse transcriptase PCR performed. Sequencing the PCR product would determine any splice variants resulting from the allele variants. There is also increasing evidence that introns contain transcriptional control elements such as enhancers or repressors (Kleinjan & van Heynigen 2005). Therefore intronic polymorphisms can also affect gene regulation. Co-transfection of a reporter gene construct, containing the 5' regulatory region, with a vector containing alternate forms of an intron would determine whether there is any alteration in the transcriptional response. Interestingly, it has been demonstrated that regulatory elements can be as far as 1Mb up- or downstream of the transcription unit (Kleinjan *et al* 2001; Lettice *et al* 2002), therefore polymorphisms within *CYP11B2* have the potential to affect the regulation of *CYP11B1* and vice versa. This would be an interesting theory to investigate. Especially to look at the influence of the IC variant in *CYP11B2* on the regulation of *CYP11B1* as many of the polymorphic alleles contributing to the IC originated in *CYP11B1*.

Four variants were identified in the 3'UTR of *CYP11B2*. Three of these were single base changes and the third was a 3bp (TCC) deletion. It is possible these variations affect RNA stability and therefore protein production. This could be tested using an *in vitro* coupled transcription/translation system followed by methods to quantify the RNA (RT-PCR) and protein (SDS-PAGE) produced and comparing the levels of the wildtype to each of the variants.

Seven single nucleotide substitutions were identified in the 5'UTR of *CYP11B2*. It is possible these could alter the transcriptional regulation of the gene, resulting in either increased or decreased expression. If this was the case, one or several of these

polymorphisms may account for the increased aldosterone levels that have previously been associated with the T/Conv haplotype. This hypothesis is to be tested in a separate study.

Of particular interest to this study is that the -344C/T and IC variants in *CYP11B2* are in strong LD with genetic variants in *CYP11B1*. Of the 19 polymorphisms identified in *CYP11B1*, only two were located within the coding sequence and both of these were the synonymous changes, Leu75Leu and Asp82Asp. As mentioned previously, the Leu75Leu polymorphism in exon 1 has been found to be in LD with the -344 C/T and IC polymorphisms in *CYP11B2* and to strongly associate with an impaired 11β-hydroylase efficiency (Keavney *et al* 2005). However, this conservative polymorphism cannot, itself, account for the biochemical change. As in *CYP11B2*, the majority of the polymorphisms were in intronic regions. The polymorphisms identified in intron 3 (2775 A/G) is located very close to the exon/intron boundary, only 12 residues away from the end of exon 3 and so may disrupt the splice site. The rest of the polymorphisms were located deeper within the introns. However, as mentioned previously, these could still potentially alter the splicing of the gene resulting in altered gene products. This requires further investigation.

Two of the identified polymorphisms were located in the 5'UTR of *CYP11B1* at positions -1889 G/T and -1859 A/G. The subsequent experimental work of this thesis focuses specifically on these two polymorphisms (see **Chapters 5 and 6**). They do not change the amino acid sequence but may change the gene's transcriptional properties, altering its basal expression or response to stimuli. They may therefore account for the observed biochemical phenotype. Most of the

CYP11B1 polymorphisms, including the -1889 G/T and -1859 A/G, show association with the T/Con haplotype, as shown in red in **table 4.2**. This is important, as it is these alleles that have previously been associated with an impaired 11β-hydroxylase efficiency.

The tight LD across the *CYP11B* locus is confirmed by the presence of relatively few common haplotypes, with 4 haplotypes (frequency greater than 5%) accounting for 68% of chromosomes in the data set (see **figure 4.9**). The T/Wt allele combination was not identified in haplotypes greater than 1%, probably due to the lower number of subjects in this group. The construction of haplotypes should enable the identification of 'tagging SNPs', to account for the observed haplotypes, allowing high throughput, efficient genotyping of the entire *CYP11B* locus. Although the sample size was small (26 subjects), previous work revealed that sample sizes of 23 and 24 individuals provide a better than 99% chance of detecting SNPs of 5% frequency, and better than 87% chance for SNPs of 1% frequency (Crawford *et al* 2004). If this is correct, our sample size is sufficient for accurate determination of haplotypes. However, the sample group in this study was selected according to their -344 C/T and IC genotypes and so confirmation of these haplotypes is required in a larger unselected group, more representative of the general population. The purpose of the haplotype analysis in the present study was to determine wherther or not and to what extent haplotypes existed across B1/B2. Further studies are currently underway to define these haplotypes in unselected populations of normotensive and hypertensive subjects. This will allow a comparison of the frequencies of the common haplotypes to see if any are associated with the hypertensive phenotype.

This study strongly suggests that the impaired 11β-hydroxylase efficiency associated with the T/Con haplotype in *CYP11B2* is due to linkage with genetic variants in *CYP11B1*. In particular, two novel single nucleotide substitutions (-1889 G/T and -1859 A/G) in the 5'UTR of *CYP11B1* have been identified as key candidates due to their potential effects on gene expression. These polymorphisms will be investigated further to assess their frequency and phenotypic associations in a large hypertensive population as well as their functional effects *in vitro*.

CHAPTER 5

Investigation of the Frequency and Phenotypic Associations of the *CYP11B1* 5'UTR Polymorphisms in a Hypertensive Population

5.1 Introduction

The SF1 -344 C/T polymorphism in *CYP11B2* has been previously associated with various cardiovascular phenotypes including hypertension with a raised ARR, increased plasma aldosterone levels as well as increased levels of its principle urinary metabolite tetrahydroaldosterone (THAldo) and left ventricular hypertrophy (LVH) (Brand *et al* 1998; Davies *et al* 1999; Delles *et al* 2001; Paillard *et al* 1999). However, these findings have not been consistently reported in all studies so that other investigators have reported no association or even an association with the contrasting allele (Kupari *et al* 1998; Pojoga *et al* 1998; Schunkert *et al* 1999a; Tsujita *et al* 2001). In contrast, one of the most consistent observations appears to be an association with reduced 11β-hydroxylase efficiency (Davies *et al* 2001; Hautanena *et al* 1998; Kennon *et al* 2004). However, the molecular basis for this is unclear, given that the biochemical phenotype is due to altered activity of the protein encoded by the adjacent *CYP11B1* gene. As the -344 C/T polymorphism has been shown to have no effect *in vitro* it seems likely that it is in linkage with other polymorphisms that directly account for the observed phenotypes (Bassett *et al* 2002). The study outlined in **Chapter 4** established that there is strong linkage disequilibrium across the entire locus encompassing the 11β-hydroxylase (*CYP11B1)* and aldosterone synthase (*CYP11B2*) genes. Two novel polymorphisms were identified in the 5'UTR of *CYP11B1* (-1889 G/T and -1859 A/G) as potential candidates for the impaired 11β-hydroxylase efficiency previously associated with the -344 T allele. These were selected as key functional candidates due to their location in the 5' regulatory region of *CYP11B1* and their potential effects on enzyme transcription. It was therefore of interest to determine whether or not these polymorphisms do indeed associate with 11β-hydroxylase efficiency.

5.2 Aims

The aim of this study was to investigate the frequency and phenotypic associations of the -1889 G/T and -1859 A/G *CYP11B1* polymorphisms in a large hypertensive population. In particular, to focus on markers indicating impaired 11β-hydroxylase efficiency and their association with genotype.

5.3 Methods

5.3.1 Study subjects

A large hypertensive study population was derived from the MRC BRItish Genetics of HyperTension (BRIGHT) study. The Bright Study is a large multicentre investigation into the genetic basis of hypertension, comprising > 2000 affected sibling pairs and trios. The primary aim of the study was to collect a large collection of affected sibling pairs to permit a genome wide scan to identify loci that might be involved in the development of hypertension. In this study, a range of phenotypes, including body mass index (BMI), blood chemistry and corticosteroid metabolite excretion were also measured. Hypertensive subjects were recruited from primary care with entry to the study being dependent on a pre-treatment blood pressure of >145/95mmHg (mean of 3 readings). All had the onset of hypertension before 60 years of age. Subjects with a BMI > 30 were excluded from the study. Full details of the recruitment strategy have been published elsewhere (Caulfield *et al* 2003).

For the present investigation, a random subgroup of 512 unrelated hypertensive subjects was selected for the analysis of urinary corticosteroid metabolite excretion rates. Full demographic information (as medians and inter-quartile ranges) on the

subjects included is shown in **table 5.1**. Ethical approval for the study was granted by the local ethics committees of the partner institutes and fully informed written consent from all of the participants was obtained.

5.3.2 Genotyping of the CYP11B1 5'UTR Polymorphisms

The entire BRIGHT study population (n = 3599) was genotyped for the -1889 G/T and -1859 A/G polymorphisms using a nested PCR method to increase specificity.

Nested PCR protocol

The initial reaction amplified a 2kb region specific to *CYP11B1* and the second reaction used this product as a template to amplify the region of interest (~400bp) specifically (see **section 2.3.2**). In both reactions, a heat-activated thermostart Taq polymerase (Abgene, UK) was used. The sense and anti-sense primers (MWG-Biotech, Ebersberg, Germany) used in both reactions are outlined in **appendix 2, table 7.**

5.3.3 Urinary Corticosteroid Metabolite Measurements

Miss Mary C Ingram performed all the urinary corticosteroid measurements on the subset of the BRIGHT population (n = 512). Urine samples (24 hour) were collected in plain plastic containers without preservative. Aliquots of urine were stored at -20°C. Excretion rates of steroid metabolites were measured by gas chromatography/mass spectrometry using the method of Shackleton (Shackleton 1993) with minor modifications. The ratio of tetrahydrodeoxycortisol/total cortisol (THS/F) was used as an index of 11β-hydroxylase activity.

5.3.4 Statistics

Whether the allele frequencies of -1889 G/T and -1859 A/G within the BRIGHT subgroup were in Hardy Weinberg equilibrium was determined using a Chi-squared test. The demographic and steroid excretion data did not follow a normal distribution and so were compared by non-parametric methods (Mann-Whitney test).

5.4 Results

5.4.1 Genotyping of -1889 G/T and -1859 A/G

Following optimisation of the nested PCR conditions each of the BRIGHT study samples, which had been pre-plated onto 96-well PCR plates (25ng/well), were amplified. This produced a PCR amplicon of 393bp as determined by agarose gel electrophoresis of a random selection of samples (12 to 16) from each plate (see **figure 5.1**). The PCR products were then sequenced (see **section 2.2.6**) using the primer B1prom-1750(as) (see **table 6, appendix 2**) to determine the -1889 and -1859 genotypes.

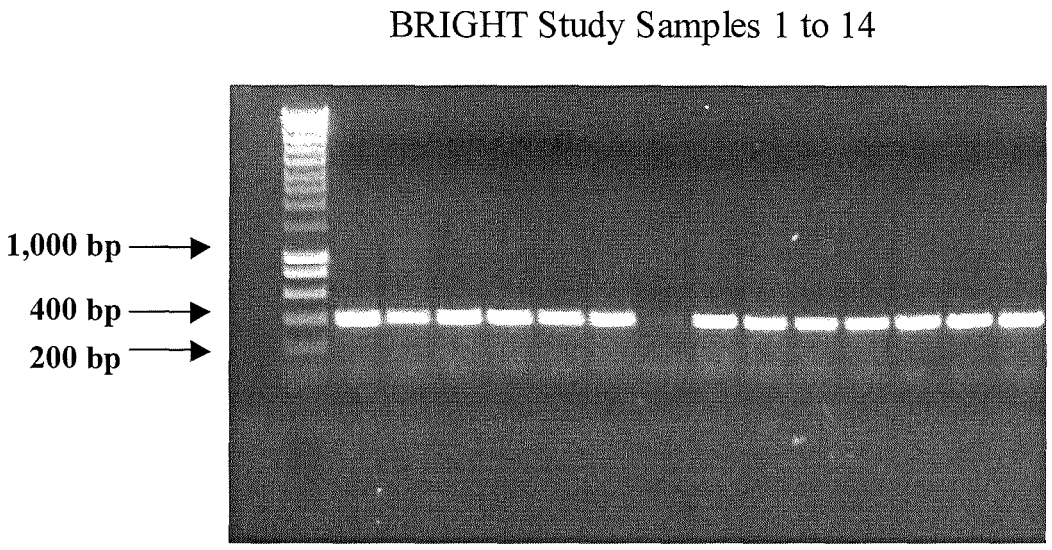


Figure 5.1. Nested PCR fragments (~ 400 bp) from 14 random BRIGHT samples, resolved on a 1% agarose gel. Lane 1 contains 5µl HyperLadder I (Bioline, London, UK). Individual bands of the correct size indicate specificity. The lack of a band in lane 7 indicates a failure. In general the failure rate for each plate was approximately 10%.

5.4.2 Demographic data for BRIGHT sub-group

Blood pressure and other relevant demographic data are shown in **table 5.1**; the population was significantly hypertensive but not significantly overweight. The only difference in characteristics of subject groups separated according to the -1889 or -1859 SNPs was with waist/hip ratio (-1889 GG vs. TT $p = 0.015$; -1859 AA vs. GG $p = 0.013$).

A)

	ALL SUBJECTS n=437	-1889 GG SUBJECTS n=116	-1889 GT SUBJECTS n=223	-1889 TT SUBJECTS n=98	p*
Age (years)	64 (57-70)	63(56-68)	64(58-71)	63(56-70)	NS
Mean SBP (mm/Hg)	181(153-191)	157(153-190)	183(153-193)	157(152-190)	NS
Mean DBP (mm/Hg)	103(98-110)	104(100-110)	103(98-110)	103(99-110)	NS
BMI (kg/m^2)	27(25-30)	27(24-30)	28(26-30)	27(25-30)	NS
WHR	0.88 (0.81-0.93)	0.84 (0.80-0.91)	0.88 (0.81-0.94)	0.89 (0.83-0.94)	**0.015**
LVmass index	68 (58-78)	69 (58-79)	67 (58-78)	67 (57-76)	NS

B)

	ALL SUBJECTS n=437	-1859 AA SUBJECTS n=106	-1859 AG SUBJECTS n=225	-1859 GG SUBJECTS n=106	p**
Age (years)	64 (57-70)	63(56-68)	64(59-71)	63(56-70)	NS
Mean SBP (mm/Hg)	181(153-191)	157(153-190)	183(153-193)	169(153-190)	NS
Mean DBP (mm/Hg)	103(98-110)	104(100-110)	103(98-110)	103(99-110)	NS
BMI (kg/m^2)	27(25-30)	27(24-30)	28(26-30)	27(25-30)	NS
WHR	0.88 (0.81-0.93)	0.84 (0.80-0.91)	0.88 (0.81-0.94)	0.89 (0.83-0.94)	**0.013**
LVmass index	68 (58-78)	69 (58-79)	67 (58-78)	67 (57-76)	NS

Table 5.1. Demographic information on BRIGHT study sub-group separated by A) -1889 and B) -1859 genotypes. In each case the data did not follow a normal distribution and so were compared by the non-parametric Mann-Whitney test.
SBP- systolic blood pressure, DBP-diastolic blood pressure, BMI-body mass index, WHR-waist-hip-ratio, LVmass-left ventricular mass.
*comparison between -1889 GG and TT homozygotes
**comparison between -1859 AA and GG homozygotes

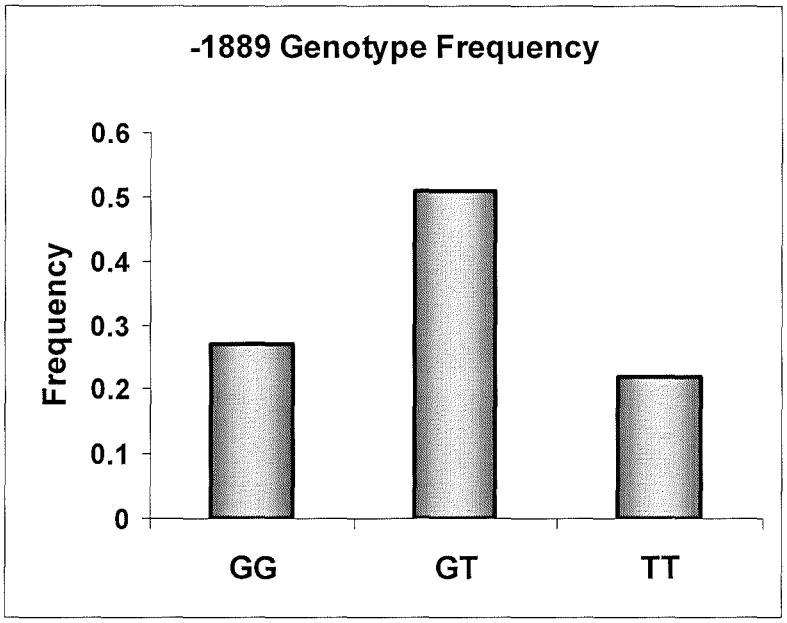
5.4.3 Genetic analysis of CYP11B1

The frequencies of the -1889 (G-0.52, T-0.48) and -1859 (A-0.50, G-0.50) polymorphisms within the BRIGHT subgroup are illustrated in **figure 5.2**. The alleles of both -1889 G/T and -1859 A/G were in Hardy-Weinberg equilibrium with Chi-square values of 0.91 and 0.82 respectively.

5.4.4 Association with THS/Total F Metabolites

The major urinary corticosteroid metabolites examined are listed in **table 5.2**. There was no significant difference in the excretion of the major aldosterone metabolite, THAldo or total androgen metabolite excretion for subjects homozygous for the -1889 G/T or -1859 A/G alleles. Importantly, there was also no significant difference in total cortisol metabolite excretion rate (nmol/24 hours) between the -1889 GG vs. TT ($p = 0.4513$) or -1859 AA vs GG ($p = 0.2789$) genotypes. The THS/ total F ratio (index of 11β-hydroxylase activity) was significantly higher in -1889 TT homozygotes than in GG homozygotes ($p = 0.025$). A similar pattern was seen with the -1859 SNP, with GG homozygotes tending to have a higher ratio than AA homozygotes ($p = 0.056$) (**table 5.2, figure 5.3**). **Figure 5.3** summarises the means of the THS/F ratios in all three genotype groups for both SNPs. There was no statistically significant difference between the -1889 TT and GT groups but the THS/F ratio remained significantly higher in the heterozygous group than the GG subjects (0.019). A similar pattern is seen for -1859, with no statistical difference between the GG and AG groups but a trend to a higher ratio in the heterozygous group than the AA homozygotes ($p = 0.066$).

A



B

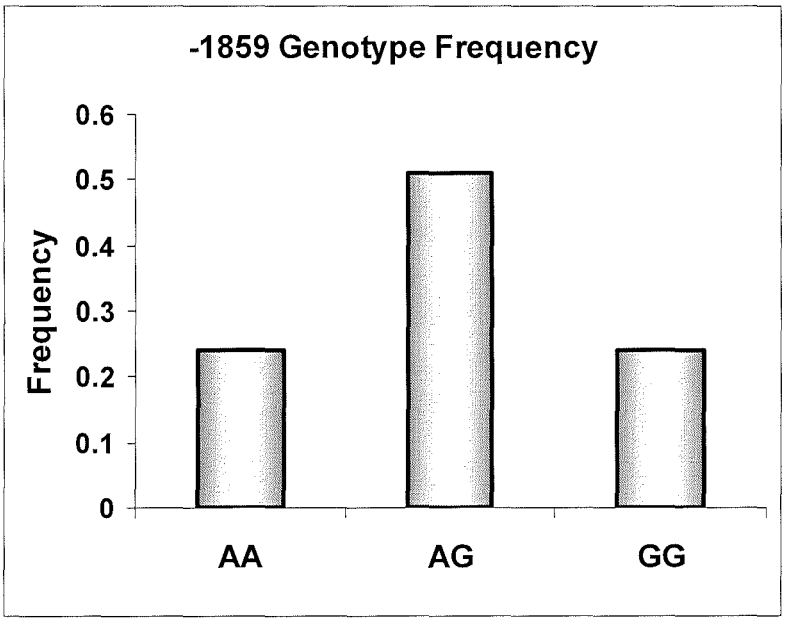


Figure 5.2. Frequencies of A) the -1889 G/T and B) the -1859 A/G polymorphisms in the BRIGHT population subgroup.

A)

Urinary Steroid Metabolites (nmoles/24hrs)	ALL SUBJECTS	-1889 GG SUBJECTS	-1889 GT SUBJECTS	-1889 TT SUBJECTS	p*
ALDOSTERONE (THAldo)	3 (2-6)	3 (2-6)	3 (2-6)	3 (1-5)	NS
CORTISOL METABOLITES (TOTAL F; THF+aTHF+THE)	1416 (788-2670)	1305 (652-2532)	1635 (835-2824)	1385 (793-2701)	NS
ANDROGEN METABOLITES (TOTAL ANDRO; DHA+AETIO+ANDRO)	606 (318-1208)	663 (276-1322)	580 (322-1209)	550 (340-1062)	NS
11β-HYDROXYLASE EFFICIENCY (THS/TOTAL F)	0.009 (0.005-0.014)	0.007 (0.005-0.012)	0.010 (0.006-0.015)	0.011 (0.006-0.015)	**0.024**

B)

Urinary Steroid Metabolites (nmoles/24hrs)	ALL SUBJECTS	-1859 AA SUBJECTS	-1859 AG SUBJECTS	-1859 GG SUBJECTS	p**
ALDOSTERONE (THAldo)	3 (2-6)	3 (1-6)	3 (2-7)	3 (1-5)	NS
CORTISOL METABOLITES (TOTAL F; THF+aTHF+THE)	1416 (788-2670)	1245 (718-2314)	1647 (798-2754)	1399 (840-2705)	NS
ANDROGEN METABOLITES (TOTAL ANDRO; DHA+AETIO+ANDRO)	606 (318-1208)	636 (257-1317)	580 (317-1213)	630 (358-1062)	NS
11β-HYDROXYLASE EFFICIENCY (THS/TOTAL F)	0.009 (0.005-0.014)	0.007 (0.005-0.012)	0.009 (0.006-0.015)	0.010 (0.005-0.016)	**0.056**

Table 5.2. Urinary corticosteroid excretion separated according to A) -1889 and B) -1859 genotypes

THF: tetrahydrocortisol, (a)THE (allo) tetrahydrocortisone,

DHA: dehydroepiandrosterone, Aetio: aetiocholanolone, Andro: androsterone THAldo:tetrahydroaldosterone, THS: tetrahydrodeoxycortisol, NS = non significant. Steroid results were compared by the non-parametric Mann-Whitney test.

* comparison between -1889 GG and TT homozygotes

**comparison between -1859 AA and GG homozygotes

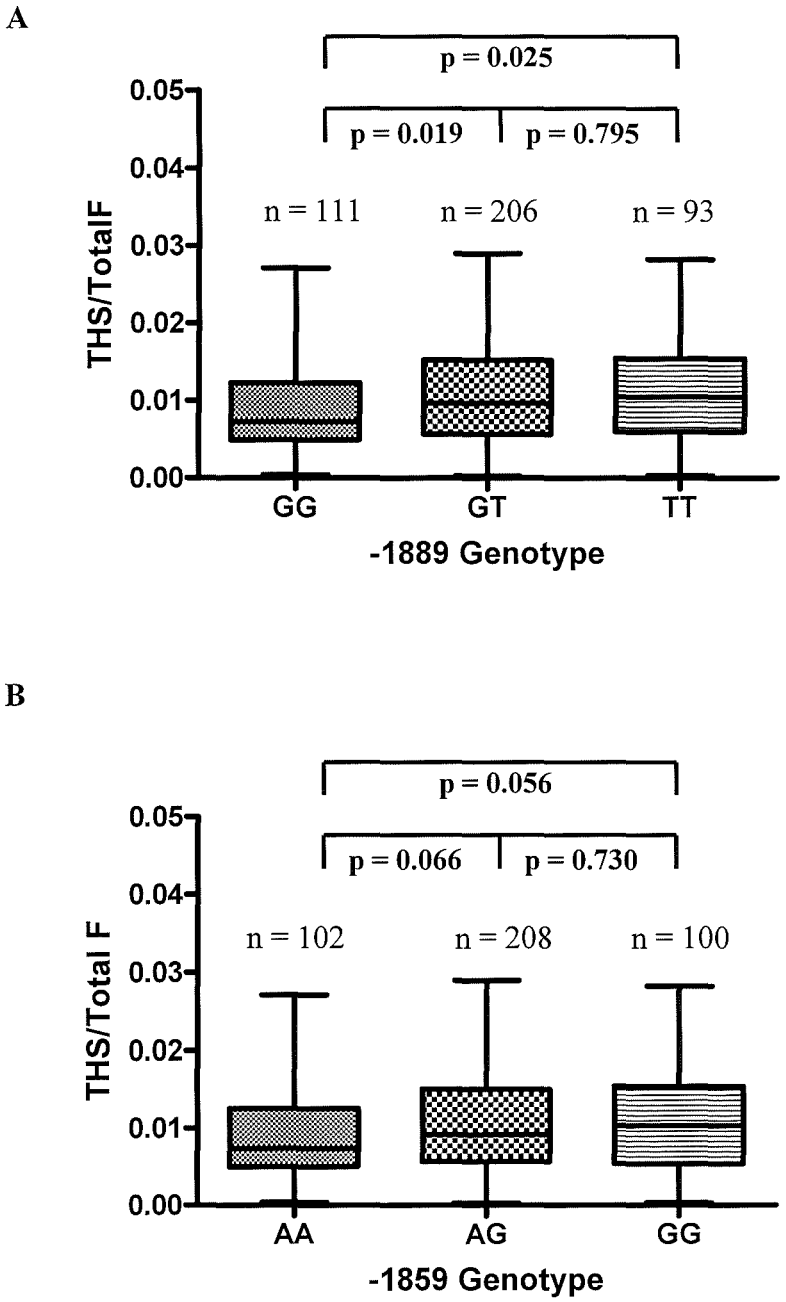


Figure 5.3. Box-whisker plots of 11β-hydroxylase efficiency (THS/total F ratio) in all subjects stratified by the -1889 or -1859 genotypes.

In each case comparisons of the tetrahydrodeoxycortisol (THS)/total cortisol (F) ratio between the genotype groups was analysed by non-parametric testing (Mann-Whitney).

5.5 Discussion

A consistent yet somewhat paradoxical association exists between variants in the *CYP11B2* gene (-344 C/T and IC) and markers of 11β-hydroxylase activity, which is the enzyme encoded by the highly homologous *CYP11B1* gene (Davies *et al* 2001; Hautanena *et al* 1998; Kennon *et al* 2004). It has been postulated that this is due to these *CYP11B2* variants being in linkage disequilibrium (LD) with polymorphisms in *CYP11B1*. Some previous studies (see **section 1.16.1**) identified polymorphisms in LD between *CYP11B1* and *CYP11B2* but none could explain the biochemical phenotype. Therefore, in this study (see **Chapter 4**) the pattern of variation across the region was investigated in greater detail.

The study in **Chapter 4** confirmed that the overall sequence variation across the *CYP11B* locus is high and that there is strong LD across the entire region. Two interesting novel polymorphisms were identified in the 5'UTR of *CYP11B1* at positions -1889 G/T and -1859 A/G that may alter transcriptional regulation of the *CYP11B1* gene and therefore affect 11β-hydroxylation efficiency, the phenotype closely associated with the T/Conversion haplotype in *CYP11B2*.

This study of an unselected hypertensive population clearly demonstrates a significant association between these polymorphisms and the ratio of THS/total F metabolites, the index of impaired 11β-hydroxylase efficiency. Those subjects homozygous for the -1889 T allele showed lower 11β-hydroxylation efficiency (increased urinary THS/total F ratio) compared with those homozygous for the G allele. Similarly, subjects homozygous for the -1859 G allele had reduced 11β-hydroxylation efficiency compared with those homozygous for the A allele. As

mentioned previously, it is these alleles (-1889 T and -1859 G) that are in linkage disequilibrium with the *CYP11B2* T/Con haplotype. Therefore, these results are in agreement with previous studies that found an association between the -344 T and IC conversion alleles and an impaired 11β-hydroxylase efficiency. Importantly, there was no difference in urinary excretion of total cortisol metabolites between the two genotype groups for each SNP (see **table 5.2**). These findings support the hypothesis that, in -344 TT subjects, normal cortisol production is maintained by a subtle increase in ACTH drive to the adrenal cortex, which increases the availability of its deoxyprecursor 11-deoxycortisol (Connell *et al* 2003).

Reduced 11β-hydroxylase efficiency is a common feature of hypertensive subjects. In 1985, de Simone and colleagues found a higher ratio of DOC/corticosterone and 11-deoxycortisol/cortisol in hypertensive subjects compared to normotensive controls after ACTH stimulation (de Simone *et al* 1985). More recently, we have made a similar observation in hypertensive subjects in whom the plasma ratio of 11-deoxycortisol/cortisol was elevated (Connell *et al* 1996). However, until now, the underlying genetic basis of this observation has not been explored. The results from the BRIGHT study confirm this phenotype of altered 11β-hydroxylation efficiency within a sub-group from a large, severely hypertensive cohort separated according to genotype at –1889 and –1859 of *CYP11B1* (11β-hydroxylase). However, there was no significant difference in mean blood pressure between the genotype groups when the cohort was separated according to the -1889 and -1859 polymorphisms. This is perhaps not surprising as all the BRIGHT study subjects are significantly hypertensive (within the upper 5% of the blood pressure range) and so it seems unlikely that a predisposition to higher blood pressure would be detected.

There was no association found between the -1889 and -1859 polymorphisms and urinary excretion levels of THAldo. Our group has previously reported an association between the *CYP11B2* -344 T and IC conversion alleles and increased urinary excretion of THAldo (Davies *et al* 1999). Therefore, as the -1889 T and -1859 G alleles in *CYP11B1* are in LD with these *CYP11B2* alleles, a similar association with THAldo might be observed. However, this was not the case (see **table 5.2**). A probable explanation for this is that many of the BRIGHT study subjects may be on antihypertensive medication that could alter their aldosterone levels including diuretics, ACE inhibitors and Ang II receptor blockers. Therefore, a more detailed analysis of a large case-control population, ideally of untreated hypertensives, is required to evaluate fully the contribution of variation across the *CYP11B* locus to aldosterone levels and hypertension and also the link between these phenotypes and the observed reduction in 11β-hydroxylase efficiency.

Aldosterone is known to promote cardiac hypertrophy and fibrosis (Brilla *et al* 1990) (see section 1.9.1.4). Patients with primary aldosteronism are more likely to have left ventricular hypertrophy (LVH) than age- and sex- matched subjects with essential hypertension of similar severity (Rossi *et al* 1997; Tanabe *et al* 1997). There have been conflicting reports of an association between the -344 C/T polymorphism and LVH (Delles *et al* 2001; Kupari *et al* 1998; Schunkert *et al* 1999a). Because the -344 and IC polymorphisms have previously been asssociated with increased aldosterone levels, the relationship between -1889 and -1859 genotype and LV mass was also investigated. No significant association with LV mass was found. This is perhaps not surprising as there was no association with aldosterone levels either. Also there was an incomplete data set, as many subjects did not have ECG criteria to

enable determination of their LV mass, resulting in a small sample size (n = 233). Again, analysis of a large case-control population would allow assessment of the association of genotype with hypertension and intermediate phenotypes such as aldosterone levels and markers of cardiovascular function such as LV mass.

An interesting association was found between the -1889 G/T and -1859 A/G polymorphisms and waist/hip ratio (see **table 5.2**). Those subjects homozygous for the -1889 T allele showed higher waist/hip ratio than those homozygous for the G allele ($p = 0.015$). Similarly, subjects homozygous for the -1859 G allele had higher waist/hip ratios than those homozygous for the A allele ($p = 0.013$). The reason for this association is not entirely clear. An increased waist/hip ratio indicates an increased centralization of body fat stores. The positive correlation between cortisol and waist/hip ratio is well established (Fraser *et al* 1999; Marin *et al* 1992). This is highlighted by the phenotype of extreme cortisol excess (Cushing's disease) where there is grossly increased central obesity (see **section 1.10.1**). However there was no significant difference in cortisol levels between the -1889 or -1859 genotypes (see **table 5.2**). It is these same alleles (-1889 T and -1859 G) that were also found to associate with an impaired 11β-hydroxylase efficiency and so cortisol levels may be maintained due to an mildly increased ACTH drive to the adrenal. Two possible explanations may therefore be considered. Firstly, a slight increase in ACTH production may have effects on the levels of other steroids such as the adrenal androgens. Obesity is known to be associated with variations in adrenal androgen synthesis, metabolism and action that differ according to sex. A recent study demonstrated an increased ACTH level in obese subjects and that this increased activity of the HPA axis correlates positively with androgen levels in obese women whereas the reverse association was observed for obese male subjects (Vicennati *et*

al 2006). The explanation for these observations remains unclear. The second possible explanation is that sites of origin for corticosteroids other than the adrenal cortex might be affected by genetic variation in *CYP11B1*. It is of interest that there is increasing evidence of extra-adrenal expression of steroidogenic enzymes such as 11β-hydroxylase (Davies & MacKenzie 2003) and the *CYP11B1* gene was recently found to be transcribed in omental but not subcutaneous adipose tissue of the rat (MacKenzie *et al* 2006). However, both its regulation and its physiological significance remain to be determined, but it could be speculated that the -1889 T and -1859 G variants are associated with altered expression of *CYP11B1* in fat tissue resulting in an increased local production of corticosteroids.

In summary, this study confirms that the -1889 G/T and -1859 A/G polymorphisms located in the 5' regulatory region of *CYP11B1* associate with 11β-hydroxylase efficiency in a hypertensive population. It seems likely that the reduced 11β-hydroxylase efficiency previously associated with the -344 T and IC conversion alleles in *CYP11B2* is due to linkage with the -1889 T and -1859 G alleles in *CYP11B1*. However, *in vitro* expression studies are required to determine whether the -1889 and -1859 polymorphisms have functional effects on *CYP11B1* transcriptional regulation.

CHAPTER 6

Functional Effects of the *CYP11B1* 5'UTR Polymorphisms and their Association with Hypertension

6.1 Introduction

As mentioned previously (see **section 1.16.2**), the -344 C/T and intron 2 conversion variants in the aldosterone synthase (*CYP11B2*) gene are associated with increased levels of 11-deoxycorticosterone (DOC) and 11-deoxycortisol (S) (and their urinary metabolites) (Davies *et al* 2001; Hautanena *et al* 1998; Kennon *et al* 2004). This reflects a reduced efficiency of adrenal 11β-hydroxylase and is likely to be a consequence of variation in the adjacent and highly homologous *CYP11B1* (11β-hydroxylase) gene. The studies described in **Chapter 4** confirmed that there is strong linkage disequilibrium (LD) across the entire CYP11B locus and that the -344 C/T and intron conversion variants in *CYP11B2* are in LD with several variants in *CYP11B1*. Two of these were novel polymorphisms identified within the 5'UTR at positions -1889 (G/T) and -1859 (A/G). The studies described in **chapter 5** demonstrated a significant association between these polymorphisms and the ratio of THS/total F (a marker of 11β-hydroxylase function) in a cohort of subjects from the BRIGHT study. To test whether these polymorphisms can cause differences in 11β-hydroxylase efficiency, their effects were studied *in vitro*.

6.2 Aims

The aims of this study were to determine the effects of the -1889 G/T and -1859 A/G polymorphisms on *CYP11B1* basal transcription and response to stimuli *in vitro* using a luciferase reporter gene system.

6.3 Methods

6.3.1 Investigation of Reporter Gene activity in vitro

All 4 combinations of the -1889 and -1859 alleles were created *in vitro* using the Quick-Change site-directed mutagenesis kit (Stratagene Ltd, Cambridge, UK) and the pB1-1937 plasmid as a template (**section 2.1.4**). The allele combinations of each construct and the *CYP11B2* genotypes they associate with are shown in **table 6.1**. Large scale purified preparations of the pB1-1937 wildtype, each of the mutants and the psvβGal control vector were made, analysed by restriction digestion and sequenced (**sections 2.1.1, 2.1.2, 2.2.6**). Transient transfection was carried in Y-1 adrenal cells (**section 2.4.1**). Cells were incubated with or without agonist; ACTH (1μM), dibutyryl cAMP ((Bu_2)cAMP) (1mM) or Forskolin (10μM) for 24 hours. Cell lysates were prepared and luciferase activity measured to determine reporter gene activity (**section 2.1.5**). Protein levels and β-galactosidase activity were also measured to determine cell number and transfection efficiency and the luciferase results were corrected appropriately (**sections 2.1.5** and **2.4.2**).

Construct	-1889 residue	-1859 residue	*CYP11B2* Association
1	G	A	CC/WtWt, TT/WtWt
2	G	G	Rarely seen
3	T	A	Rarely seen
4	T	G	TT/ConCon

Table 6.1 Constructs used for transfections showing their -1889 and -1859 allele combinations and the *CYP11B2* genotypes they would associate with.

6.3.2 Statistics

The *in vitro* expression results for each construct were compared to the appropriate control using an unpaired 2-sample t test. Whether the allele frequencies of -1889 G/T and -1859 A/G within the BRIGHT case-control study were in Hardy Weinberg equilibrium was determined using a Chi-squared test. The demographic characteristics were not normally distributed and so a comparison of the cases versus controls wasdetermined using a Mann-Whitney test. In all circumstances, a p value of less than 0.05 was accepted as significant.

6.3.3 Investigation of Transcription Factor Binding Sites

To investigate whether the -1889 G/T and -1859 A/G polymorphisms reside within putative transcription factor binding sites, the DNA sequence surrounding them was analysed using the Transcription Element Search Software (TESS) (http://www.cbil.upenn.edu/tess) as well as the programme MatInspector (Quandt *et al* 1995).

6.3.4 Study Subjects

The BRIGHT case-control association study comprises 800 unrelated hypertensive subjects (from the total of > 2000 affected sibling pairs and trios) with 800 age- and sex- matched normotensive control subjects. Full demographic information (as medians and inter-quartile ranges) on the subjects included is shown in **table 6.2.**

	CASES	CONTROLS	p value
AGE (years)	60 (52-66)	60 (52-66)	NS
MEAN SBP (mm Hg)	154 (141-249)	124 (116-132)	< 0.0001
MEAN DBP (mm Hg)	93 (86-101)	77 (73-82)	< 0.0001
BMI (kg/m^2)	27 (25-30)	25 (23-27)	< 0.0001

Table 6.2. Demographic information on BRIGHT case-control study sub-groups

SBP- systolic blood pressure, DBP-diastolic blood pressure, BMI-body mass index, NS – non-significant.

6.4 Results

6.4.1 Restriction Digestion of Plasmids

The pB1-1937 construct comprises the pGL3-Basic vector backbone (4.8 kb) (Promega Corp., Southampton, UK) and an insert of approximately 1.9kb. The plasmid DNA was digested using the restriction enzyme BamHI to assess quality and the presence of an insert of appropriate size. This enzyme does not cut the B1 5' UTR insert but linearizes the pGL3-Basic vector by hydrolysing it at a single site to produce a fragment of approximately 6.7kb. BamHI digests are shown in **figure 6.1**.

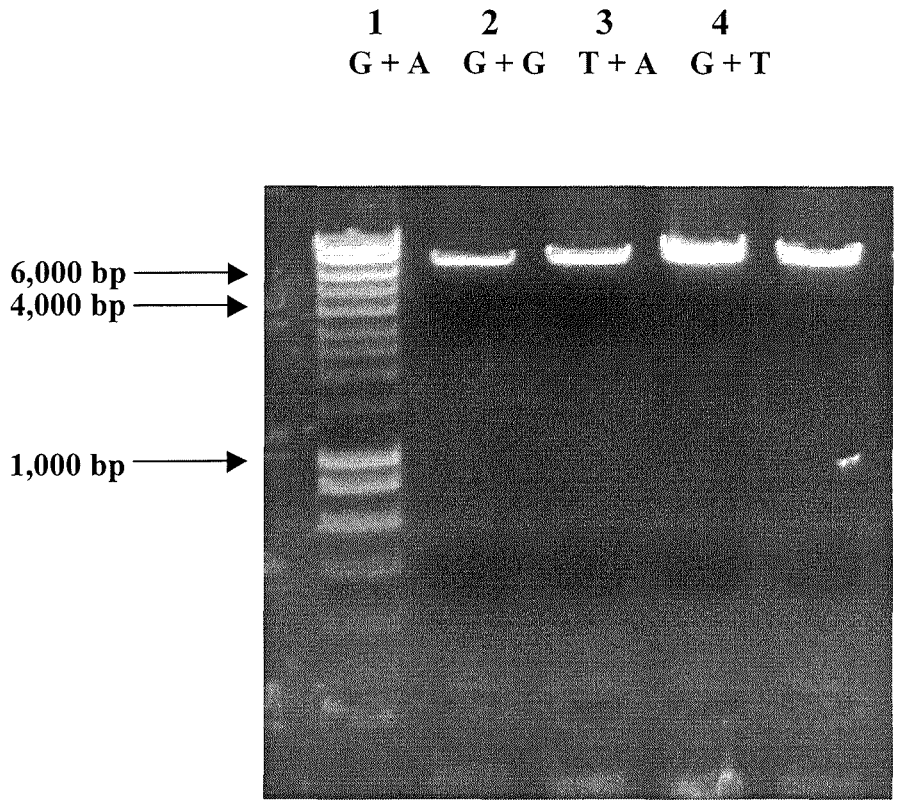


Figure 6.1. BamHI digests of pB1-1937 constructs

Analysed on a 1% agarose gel stained with ethidium bromide. BamHI linearises the pGL3 Basic vector backbone to generate a 6.7kb band. A single band of the correct size confirms the presence of the *CYP11B1* cDNA insert. Lane L contains 5 µl HyperLadder I (Bioline, London, UK).

6.4.2 Confirmation of Mutations

The presence of each of the mutants was verified by sequencing using the automated sequencing method described in **section 2.2.6**. The entire insert was sequenced to rule out the incorporation of any unwanted mutations. Sequence data are shown in **figure 6.2**.

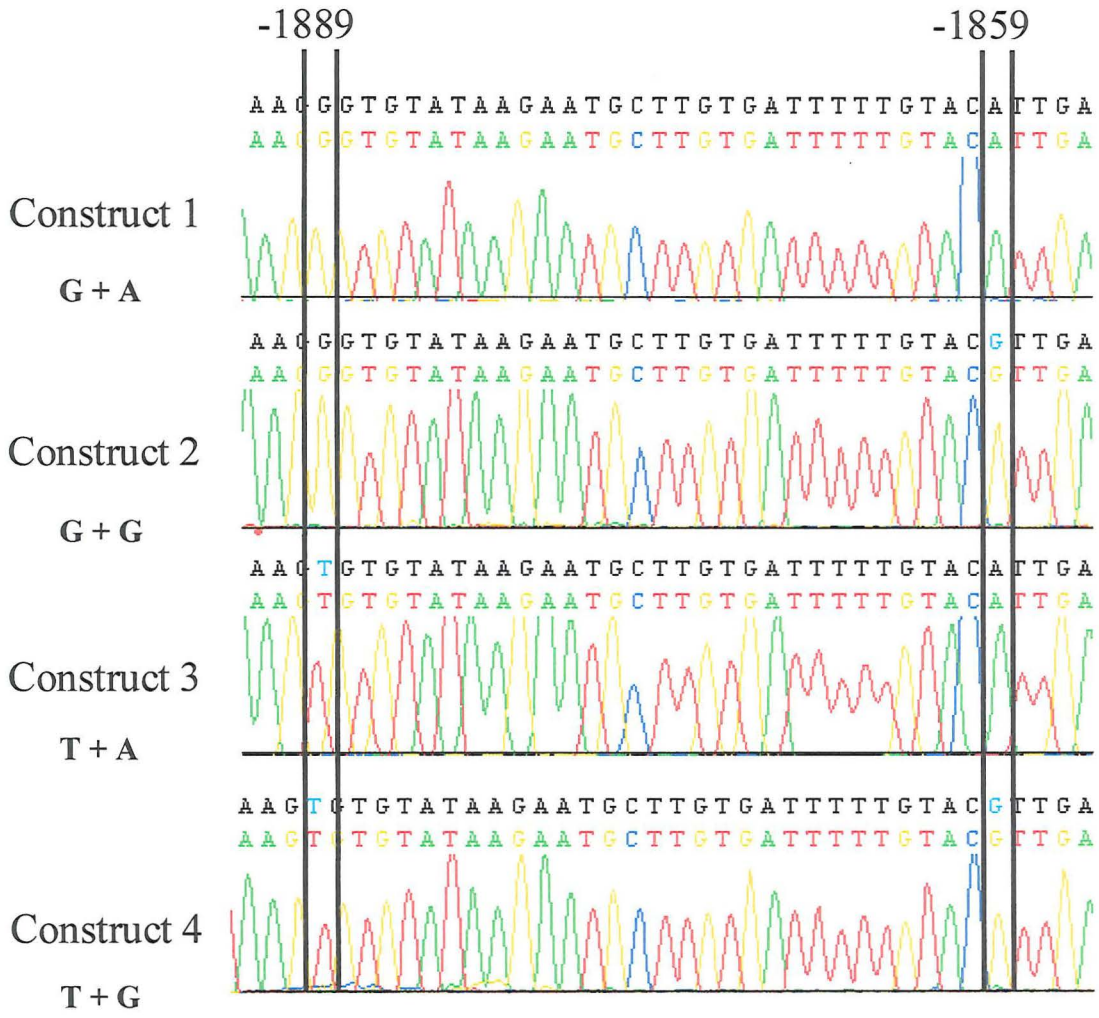


Figure 6.2. Electropherograms confirming the incorporation of the desired mutations in each of the pB1-1937 constructs.

6.4.3 Transcriptional actvity in vitro

Investigation of the -1889 G/T and -1859 A/G single base changes using luciferase reporter gene constructs revealed no significant effect on basal expression levels (**figure 6.3**). The construct containing the -1889 T and the -1859 G alleles had a significantly reduced response to stimulation with 1μM ACTH ($p = 0.001$) when compared to the wildtype allele combination -1889 G and -1859 A (**figure 6.4**). The other 2 constructs (G + G and T + A) also appeared to have a reduced response to ACTH stimulation compared to the wildtype (G + A) but this did not quite reach statistical significance (G + G $p = 0.092$, T + G $p = 0.088$). Treatment with 1mM (Bu_2)cAMP failed to stimulate reporter gene activity (**figure 6.5**). Treatment with 10μM Forskolin produced a similar pattern to that of ACTH. Construct 4 (T + G) had a significantly reduced response to forskolin compared to the wildtype construct 1 (**figure 6.6** $p = 0.025$). Again, constructs 2 (G + G) and 3 (T + A) appeared to have a slightly reduced response to stimulation compared to wildtype but this was not statistically significant (G + G $p = 0.514$, T + G $p = 0.311$).

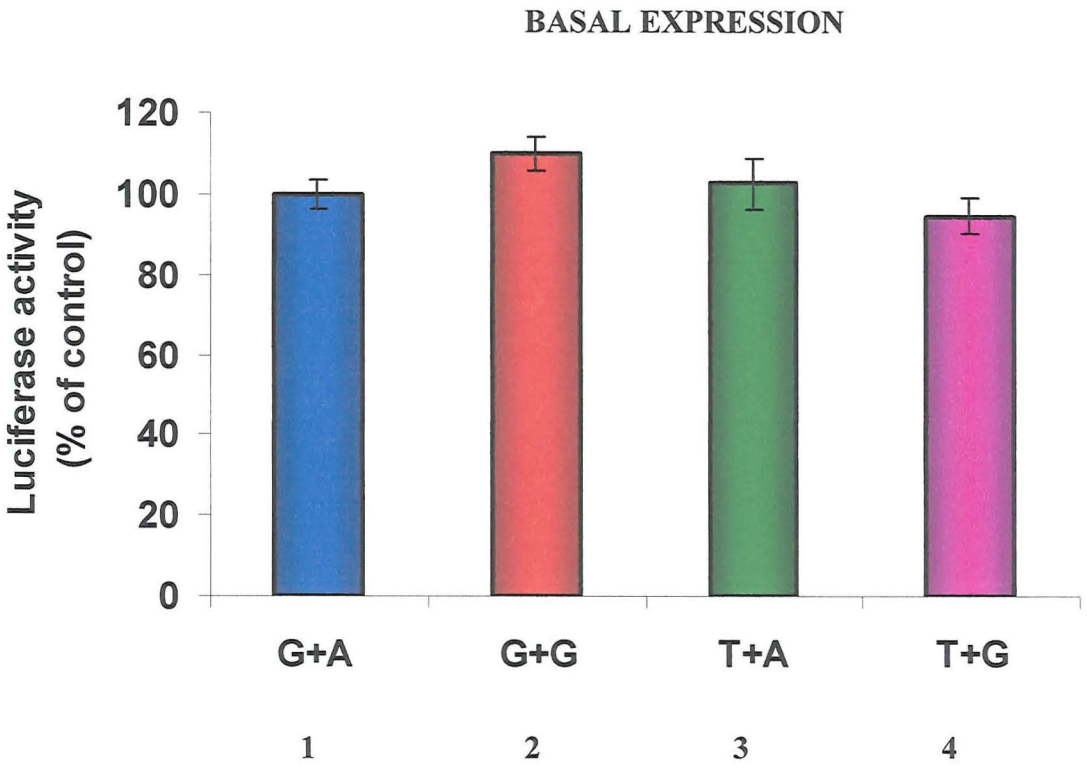


Figure 6.3. Effects of the -1889 G/T and -1859 A/G variants on basal transcriptional activity

Y-1 adrenocortical cells were transfected with luciferase reporter constructs containing pB1-1937 (1μg) with various combinations of the -1889 and -1859 alleles for 48 hours. Construct 1, referred to as the wildtype (-1889 G and -1859 A), is most commonly associated with the CC/WtWt and TT/WtWt *CYP11B2* genotypes. Construct 4 (-1889 T and -1859 G) contains the alleles found in linkage with the *CYP11B2* TT/ConCon genotype. Results are expressed as a percent of the wildtype (construct 1) activity. The results represent the mean ± SEM of data from six independent experiments, each with an n of 3 to 9. Each construct was compared to the wildtype (construct 1) using an unpaired 2-sample t test.

No significant difference was observed between the constructs under basal conditions.

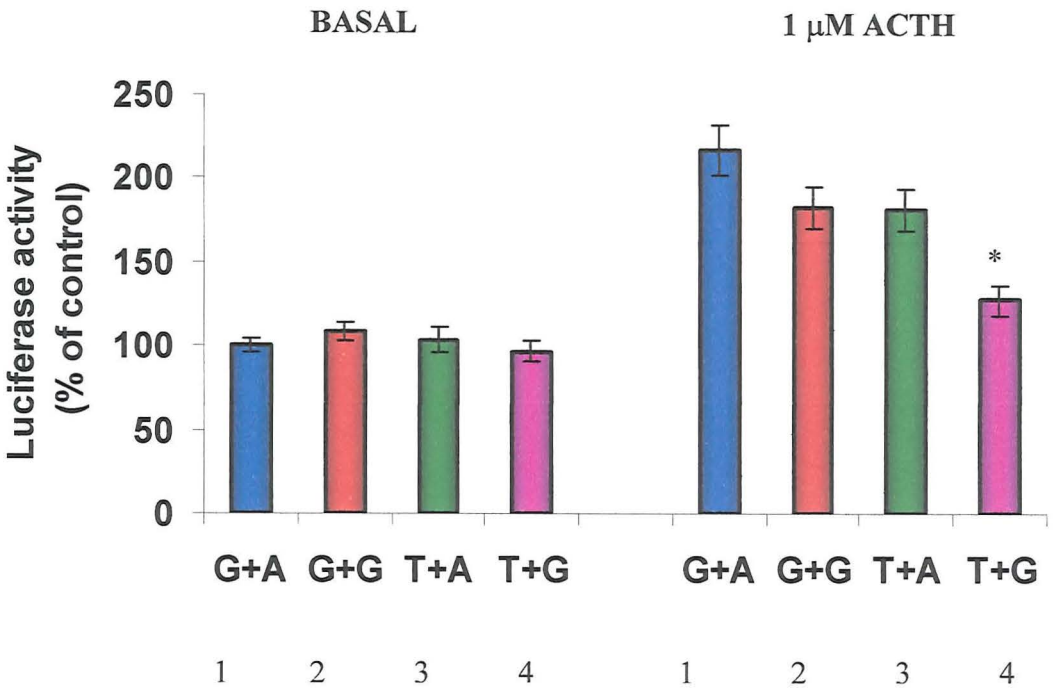


Figure 6.4. Effects of the -1889 G/T and -1859 A/G variants on transcriptional activity in response to ACTH.

Y-1 adrenocortical cells were transfected with luciferase reporter constructs containing pB1-1937 (1μg) with various combinations of the -1889 and -1859 alleles. Construct 1, referred to as the wildtype (-1889 G and -1859 A), is most commonly associated with the CC/WtWt and TT/WtWt *CYP11B2* genotypes. Construct 4 (-1889 T and -1859 G) contains the alleles found in linkage with the *CYP11B2* TT/ConCon genotype. Following transfection for 42 hours cells were then incubated with ACTH (1μM) for 6 hours. Results are expressed as a percent of the basal reporter activity of the wildtype (construct 1) and represent the mean ± SEM of data from three independent experiments, each with an n of 3 to 9. The results for each construct were compared to the appropriate control (basal or stimulated wildtype) using an unpaired 2-sample t test.

* $P < 0.001$ compared with G+A stimulated

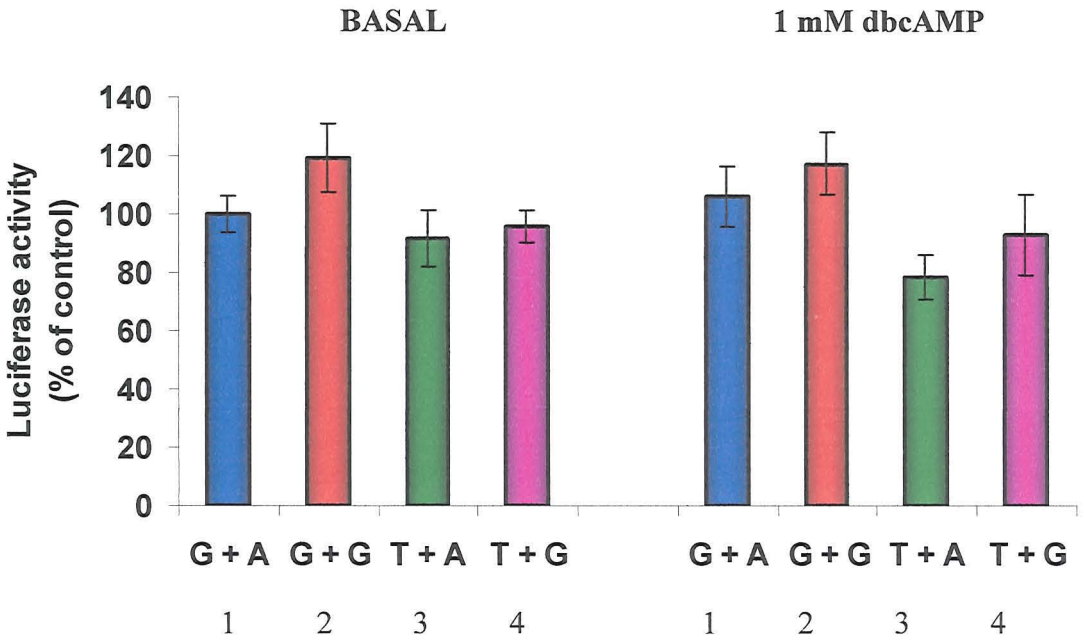


Figure 6.5. Effects of the -1889 G/T and -1859 A/G variants on transcriptional activity in response to dibutryl cAMP.

Y-1 adrenocortical cells were transfected with luciferase reporter constructs containing pB1-1937 (1μg) with various combinations of the -1889 and -1859 alleles. Construct 1, referred to as the wildtype (-1889 G and -1859 A), is most commonly associated with the CC/WtWt and TT/WtWt *CYP11B2* genotypes. Construct 4 (-1889 T and -1859 G) contains the alleles found in linkage with the *CYP11B2* TT/ConCon genotype. Following transfection for 42 hours cells were then incubated with dbcAMP (1mM) for 6 hours. Results are expressed as a percent of the basal reporter activity of the wildtype (construct 1) and represent the mean ± SEM of data from two independent experiments with an n = 3 and n = 6 respectively. The results for each construct were compared to the appropriate control (basal or stimulated wildtype) using an unpaired 2-sample t test.

No significant difference was observed between any of the constructs stimulated with dbcAMP.

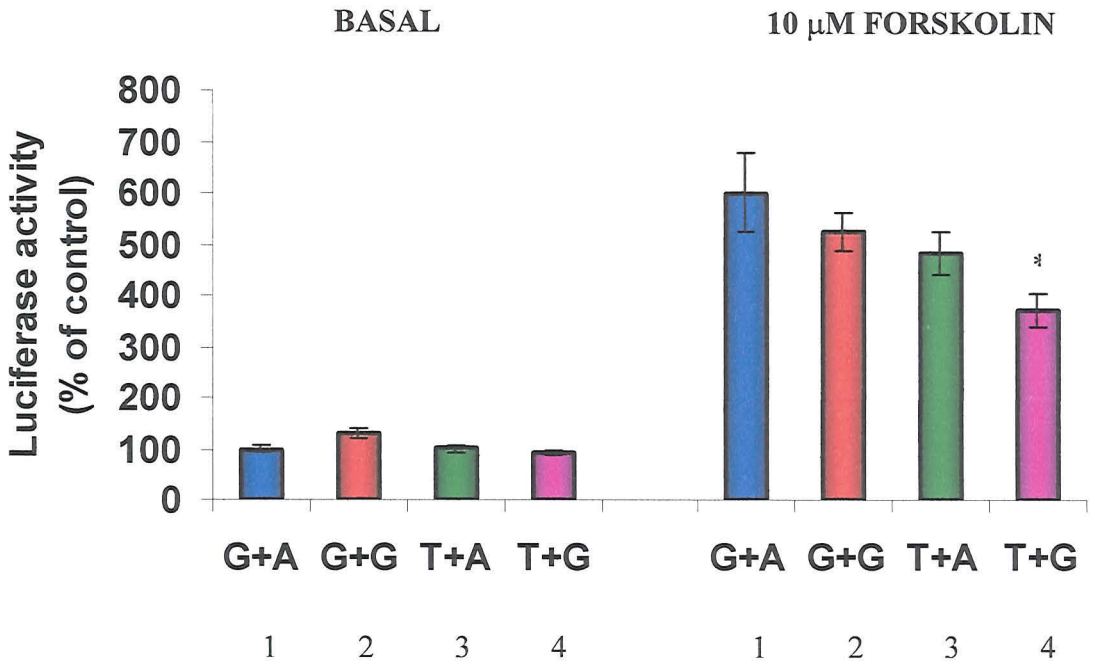


Figure 6.6. Effects of the -1889 G/T and -1859 A/G variants on transcriptional activity in response to Forskolin.

Y-1 adrenocortical cells were transfected with luciferase reporter constructs containing pB1-1937 (1μg) with various combinations of the -1889 and -1859 alleles. Construct 1, referred to as the wildtype (-1889 G and -1859 A), is most commonly associated with the CC/WtWt and TT/WtWt *CYP11B2* genotypes. Construct 4 (-1889 T and -1859 G) contains the alleles found in linkage with the *CYP11B2* TT/ConCon genotype. Following transfection for 42 hours cells were then incubated with forskolin (10 μM) for 6 hours. Results are expressed as a percent of the basal reporter activity of the wildtype (construct 1) and represent the mean ± SEM of data from three independent experiments, each with an n of 3 to 9. The results for each construct were compared to the appropriate control (basal or stimulated wildtype) using an unpaired 2-sample t test.

* P = 0.036 compared with G+A stimulated

6.4.4 Transcription Factor Binding Sites

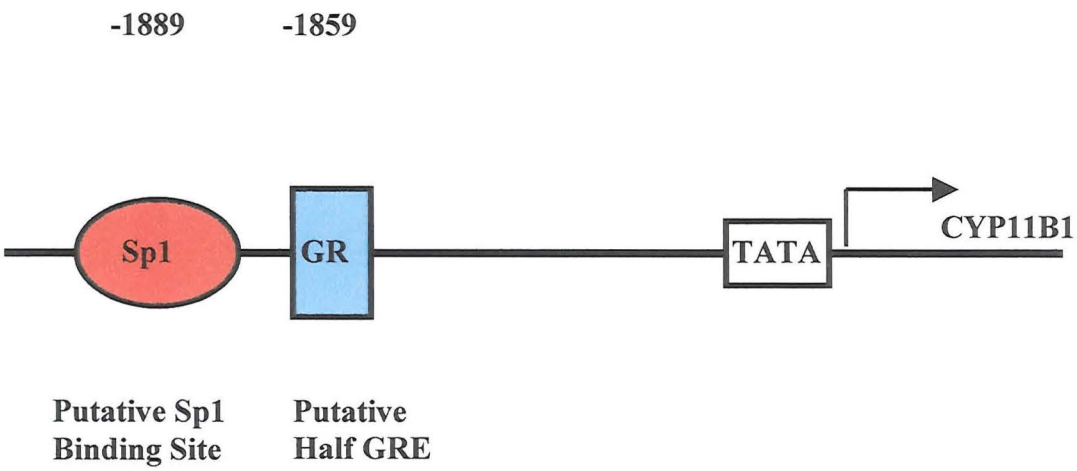
Analysis of the sequence surrounding the -1889 G/T and -1859 A/G polymorphisms revealed that they disrupt several putative transcription factor binding sites. Comparison of the likelihood scores of the identified binding elements and investigation of which transcription factors were most likely to be found in steroidogenic tissues suggested that the most likely binding sites were an Sp1 site encompassing residue -1889 and a half GRE encompassing residue -1859 (**figure 6.7**).

6.4.5 Frequency of -1889 G/T and -1859 A/G in BRIGHT case-controls

The allele frequencies for both -1889 G/T (0.546/0.454 vs. 0.562/0.438) and -1859 A/G (0.524/0.476 vs. 0.527/0.473) were almost identical in the hypertensive and normotensive control groups (see **table 6.3**). The alleles of both -1889 G/T and -1859 A/G were in Hardy-Weinberg equilibrium in both groups, with Chi-square values of 0.002 and 0.04 respectively in the hypertensive group and 3.79 and 1.04 respectively in the control group.

	-1889 G	**- 1889 T**	**- 1859 A**	**- 1859 G**
CASES	0.546	0.454	0.524	0.476
CONTROLS	0.562	0.438	0.527	0.473

Table 6.3. Allele frequencies of -1889 G/T and -1859 A/G in the BRIGHT case-control association study



Transcription Factor	Binding Sequence
-1864/-1859 GR	TGTACA G
-1891/-1882 Sp1	AGGGcGTATA T

Figure 6.7. Map of the novel putative transcription factor binding sites in the h*CYP11B1* 5'UTR. The blue A and G represent the -1859 polymorphism and the red G and T represent the -1889 polymorphism.

6.5 Discussion

The two novel single nucleotide polymorphisms (SNPs) identified in the 5'UTR of *CYP11B1* at positions -1889 (G/T) and -1859 (A/G) are in linkage disequilibrium (LD) with the -344 C/T and intron conversion variants in *CYP11B2*. These have the potential to alter transcriptional regulation of the *CYP11B1* gene and therefore may account for the observed biochemical phenotype of decreased 11β-hydroxylation.

This study investigated the effects of the -1889 G/T and -1859 A/G SNPs on *CYP11B1* gene transcription *in vitro* using a luciferase reporter gene system. The variants at each locus were tested individually and in combination. Their effects on basal expression as well as the response to various agonists were examined. No significant difference was observed between any of the constructs' basal expression levels. This is perhaps not surprising as a previous study examining the transcriptional regulation of human *CYP11B1* gene indicated that the DNA region between -214 and -65 was sufficient for basal gene expression (Wang *et al* 2000). Regions upstream of this (up to -1102bp) had no effect on basal expression levels. Treatment with $(Bu)_2$cAMP had no effect on the reporter gene activity of any of the constructs. However, previous studies report a lack of cell responsiveness to this cAMP analogue in some Y-1 cell strains (Clyne *et al* 1996). ACTH is the main regulator of *CYP11B1* expression *in vivo*. It stimulates expression of the steroidogenic enzymes and steroid hormone production through activation of the cAMP signalling pathway. Forskolin is a small molecule agonist of adenylate cyclase which bypasses the ACTH receptor by binding directly to adenylate cyclase and increasing cAMP synthesis. Compared to 'wild type' (-1889 G and -1859 A), the construct containing the -1889 T and -1859 G alleles halved reporter gene

activity in response to the agonists ACTH and forskolin, indicating reduced transcription (see **figures 6.3 to 6.6**). The individual base changes represented by constructs 2 and 3 (G + G or T + A) also had a slightly reduced response to either ACTH or Forskolin stimulation when compared to wildtype but this did not reach statistical significance. This suggests there is an interaction between the -1889 T and -1859 G variants resulting in a reduced transcriptional efficiency. This might occur if they are located within the binding sites of co-operative transcription factors (see below). These results support the urinary steroid results from **Chapter 5**, which indicated that the -1889 T and -1859 G alleles were associated with an impaired 11β-hydroxylase efficiency. They strongly suggest that the reduced 11β-hydroxylation associated with the T/Con haplotype in *CYP11B2* is due to linkage with the -1889 T and -1859 G variants in *CYP11B1*.

To investigate why these variants are important for transcriptional regulation of *CYP11B1*, the sequence surrounding them was analysed computationally for putative transcription factor binding sites. Several possible candidates were generated. The most promising of these were a putative Sp1 (selective promoter factor 1) binding site (-1891/-1882) and a half GRE (-1864/-1859) encompassing the -1889 G/T and -1859 A/G polymorphisms respectively. Sp1 belongs to a large family of protein factors that all recognize similar sequences and have similar transactivation properties (Hagen *et al* 1992; Kingsley & Winoto 1992). Sp1 regulates the transcription of many genes; it stimulates the cAMP-dependent transcription of steroidogenic genes including bovine *CYP11A1* (P450scc), human StAR gene, *CYP17* (P450c17) and ferrodoxin (Lin *et al* 2001; Liu & Simpson 1997; Sugawara *et al* 2000; Yeh *et al* 2000). Sp1 usually binds to the DNA sequence GGGGCGG with high affinity (Letovsky & Dynan 1989). However, variant G-rich sequences that

deviate by at least one nucleotide and bind Sp1 with lower affinity seem to be sufficient for steroidogenic transcription (Yeh *et al* 2000).

The close proximity of the Sp1 binding site and the half GRE is of great interest as glucocorticoid binding sites can act synergistically with binding sites for other transcription factors, including Sp1 (Schule *et al* 1988; Strahle *et al* 1988). The significance of the half GRE is unclear. However, as mentioned in Chapter 1, steroid/GR complexes have been shown to bind composite GREs comprised of GRE and a binding site for another transcription factor such as AP1 (Diamond *et al* 1990; Pearce & Yamamoto 1993; Zhang *et al* 1991). In these instances, it is thought that both DNA binding by the steroid/GR complex and protein-protein interactions with other factors are required to determine transcription. Whether a half GRE is sufficient for steroid/GR binding and whether the half GRE and the Sp1 sites are close enough to establish an interaction remain to be determined. It will be of interest to determine whether these putative transcriptional factor binding sites are capable of altering function. Electrophoretic mobility shift assay experiments would determine whether Sp1 and a steroid/GR complex are capable of binding to these putative binding sites and whether this binding is disrupted by the presence of the -1889 T and -1859 G variants respectively.

To investigate the role of the half GRE, it would be interesting to determine the effect of the synthetic glucocorticoid, dexamethasone, on the reporter gene expression of each of the constructs. This would test whether the presence of the -1859 G variant that appears to disrupt the half GRE alters the transcriptional response. However, as there seems to be an interaction between the -1889 T and -1859 G variants following stimulation, the effect of dexamethasone on the response

to ACTH or forskolin stimulation should also be tested. It is possible that, in response to stimulation, there is an increased activation of Sp1. Therefore, it would also be interesting to investigate the effect on reporter gene expression, for each construct, of cotransfecting with an expression vector containing Sp1. The combined effect of cotransfecting with Sp1 and incubating with dexamethasone in the absence or presence of ACTH should indicate whether there is any interaction between the two proposed binding sites.

11β-Hydroxylase efficiency is mildly impaired in subjects with essential hypertension compared to controls (Connell *et al* 1996; de Simone *et al* 1985). The -344 T and IC conversion alleles have been shown to occur more frequently in hypertensive subjects, particularly those with a raised ARR (Lim *et al* 2002b). As mentioned in **Chapter 4**, the -344 C/T and IC polymorphisms are also in LD with other variants in *CYP11B2* that may also alter the relationship between aldosterone and Ang II.

To determine if the *CYP11B1* -1889 G/T and -1859 A/G polymorphisms are associated with hypertension, their frequencies were compared in a subset of the BRIGHT study with age and sex matched controls (see **table 6.2**). There was no apparent difference in allele frequencies between the hypertensive and normotensive control groups for either polymorphism (see **table 6.3**). This may indicate that, although they may account for the intermediate phenotype of reduced 11β-hydroxylase efficiency, they are not directly associated with hypertension. However, a more detailed analysis of a large case-control population using haplotype tags is required to evaluate fully the contribution of the *CYP11B* locus to hypertension. Additionally, a more detailed stratification of sub-phenotypes within essential

hypertension will be required before the contribution of single gene loci can be determined. For example, it is possible that one or more of the novel SNPs identified in *CYP11B2* have effects on enzyme activity or expression, resulting in increased aldosterone levels that have previously been associated with the T/Con haplotype. Recent reports that 10 to 15% of patients with essential hypertension have a raised ARR suggest that altered aldosterone regulation may be a relatively common feature. This study demonstrates that the -1889 G/T and -1859 A/G polymorphisms in *CYP11B1* that associate with reduced 11β-hydroxylase efficiency are in LD with those variants in *CYP11B2* that associate with higher aldosterone levels. How the two effects are related requires further investigation.

In conclusion, the -1889 G/T and -1859 A/G polymorphisms in the 5'UTR of *CYP11B1* have significant effects on gene transcription. This is the first time that single nucleotide polymorphisms in the regulatory region of *CYP11B1* have been shown to have a major effect on gene transcription. This study strongly suggests that the impaired 11β-hydroxylase efficiency previously associated with the -344 T and IC alleles in *CYP11B2* is due to linkage with these two novel polymorphisms in the 5' regulatory region of *CYP11B1*.

CHAPTER 7

General Discussion

Hypertension and cardiovascular disease are major human health issues with important yet undefined genetic components. In **Chapter 1**, the case was made that corticosteroids, and in particular aldosterone, are central influences in these processes. Aldosterone can cause both hypertension and cardiac remodelling and recent studies using the ARR as a screening tool suggest that altered aldosterone regulation may be a relatively common feature of essential hypertension (see **sections 1.9.1, 1.10.2 and 1.16**). Moreover, genetic variants in the aldosterone synthase, *CYP11B2*, gene (-344 C/T and IC) correlate both with increased aldosterone levels and raised blood pressure in many studies. However, these probably do not explain either biochemical or clinical differences for two reasons. Firstly, when tested *in vitro*, the -344 C/T polymorphism did not influence aldosterone synthesis. Secondly, the biochemical phenotype most consistently associated with these variants was altered 11β-hydroxylation efficiency, which draws attention to the homologous gene *CYP11B1* (see **section 1.16.2**). It was plausible to suggest that the association of increased aldosterone levels and reduced 11β-hydroxylase efficiency with the -344 C/T and IC variants is indirect through other linked genetic variations. The studies described in this thesis have addressed this question. Are the two phenomena, altered aldosterone levels and altered 11β-hydroxylation, separate or linked? Polymorphic variation across the entire *CYP11B2/B1* region was evaluated. The functional significance of some newly identified variations was tested. Finally, preliminary evaluation of possible clinical importance was made.

An important and unequivocal observation that can be made from these studies is that there is a high degree of sequence variation within the *CYP11B1* and *CYP11B2* genes. Despite the potential this gives for many haplotypes, the degree of linkage

disequilibrium is so great that most alleles fall within four common haplotypes. This identified many polymorphic differences in both genes that are linked to the -344 C/T and IC variants in *CYP11B2*. Therefore, if any of these polymorphisms are functional, it is possible that they could explain the biochemical and clinical changes associated with these variants.

The mutations identified in **Chapter 3**, within the coding region of *CYP11B1*, had effects on biochemical activity, causing either increased or decreased enzyme activity that in some cases was substrate specific. Although attempts were made to determine the locations of these mutants within the protein these were necessarily speculative. If the precise 3D crystal structure of 11β-hydroxylase was available, the specific effects of the different mutants could be valuable in deciphering structure/function relationships.

No clinical associations (with hypertension or ARR) could be made in this study. This could imply either that they are not relevant or that the study was not of a design to identify a relationship. The study could be improved and developed in several ways. Firstly, only the coding regions of *CYP11B1* were screened. Further mutations could exist in the untranslated regions, either 5' or 3' of the gene, or located at exon/intron boundaries that may alter splicing, which might associate with hypertension and a raised ARR. These studies are currently underway. Secondly, the mutation detection was performed by SSCP. Whilst a useful and sensitive technique, it has certain disadvantages. The method can be prone to both false positive and false negative results. In this instance, the false positives are less of a problem as the presence of mutations was confirmed by sequencing. However, false negative results mean that important mutations may have been overlooked. The more recent

introduction of very stringent, high throughput sequencing methods would remove the risk of false positive or negative results and also reduce the labour intensity if the study were to be repeated. Finally, the T variant of the -344 C/T polymorphism of the adjacent *CYP11B2* gene has previously been found in higher frequency in hypertensive subjects with a raised ARR but this was not the case in the current study. An important point is that the investigation outlined in **Chapter 3** was not powered to look for such an association; the study subjects were chosen on the basis of phenotype not genotype. It is therefore more likely that patients selected on the basis of genotype associated with a raised ARR might identify specific mutations that are linked to the development of this type of hypertension. Therefore, the rest of the experimental work of this thesis focused on examining the linkage between the –344 C/T and IC variants and other polymorphisms in *CYP11B2* and *CYP11B1* and their functional effects and clinical relevance.

Many novel variants were identified in *CYP11B2* that are linked to the –344 C/T and IC variants. Most of these were within the introns and 3'UTR of *CYP11B2* and their functional effects remain to be determined. Of particular interest was the identification of 7 SNPs within the 5' regulatory region of *CYP11B2*. Due to their potential to alter gene transcription, it is hypothesized that they may provide the link between the apparently non-functional –344 C/T polymorphism, increased aldosterone levels and hypertension with a raised ARR. Analysis of their effects *in vitro* using reporter gene constructs and investigation of their clinical associations will determine if this is indeed the case. However, these were not examined further in the present study.

An important and new finding described in this thesis is that the -344 C/T and IC variants in *CYP11B2* are in strong LD with many genetic variants in *CYP11B1*. Two novel and interesting polymorphisms were identified in the 5' regulatory region of *CYP11B1* at positions -1889 (G/T) and -1859 (A/G). These were found to associate with an impaired 11β-hydroxylase efficiency in a hypertensive population and to significantly alter the transcriptional response of *CYP11B1* to stimulation by ACTH or Forskolin *in vitro* (**Chapters 5 and 6**). Therefore, it seems plausible that it is these polymorphisms that account for the observed variations in 11β-hydroxylase efficiency *in vivo*. These studies strongly suggest that the reduced 11β-hydroxylation previously associated with the T/Con haplotype in *CYP11B2* can be explained by linkage with the -1889 T and -1859 G variants in the 5' regulatory region of *CYP11B1*.

Evidence of a slightly reduced 11β-hydroxylase efficiency is a consistent finding in subjects with essential hypertension compared to normotensive controls. However, preliminary investigation of the -1889 G/T and -1859 A/G polymorphisms in the BRIGHT case-control population failed to show any association with hypertension (see **Chapter 5**). Therefore, further studies are required to determine the link between this biochemical phenotype and hypertension. For example, a more detailed analysis of a much larger case control population with better phenotypic data would enable determination of their association with specific subgroups of hypertension such as hypertensives with a raised ARR. Also, investigation of an association between the common *CYP11B* haplotypes and hypertension would determine more fully the contribution of this region to hypertension.

These studies have provided an explanation, with good experimental evidence, for the decreased 11β-hydroxylation efficiency associated with the -344 C/T and IC variants; linkage with the *CYP11B1* -1889 G/T and -1859 A/G 5' UTR polymorphisms. Can these variants also explain the link to raised aldosterone levels? One explanation for the increased aldosterone levels and blood pressure associated with the -344 C/T is a linkage with other *CYP11B2* 5' UTR polymorphisms. Such polymorphisms have been identified here but whether they alter aldosterone synthesis remains to be determined. An alternative hypothesis that has previously been speculated, is that altered 11-hydroxylation efficiency, that is genetically determined, might give rise to a phenotype of mineralocorticoid hypertension as a result of a long-term but very mild increase in ACTH drive to the adrenal cortex, to maintain normal plasma cortisol levels (see **section 1.16.3**). The results presented in this thesis support this theory; analysis of the steroid results in the BRIGHT population according to the -1889 G/T or -1859 A/G polymorphisms revealed no significant difference in urinary cortisol metabolite excretion between genotypes but an increase in THS excretion rate in the -1889 T and -1859 G groups (see **Chapter 5**). It has been postulated that this subtle increase in ACTH drive results in an enhanced aldosterone synthetic capacity of the adrenal cortex and response to stimuli such as angiotensin II or potassium, leading to the phenotype of hypertension with a raised ARR (see **section 1.16.3**). To investigate this hypothesis, it would be interesting to compare the ACTH levels of untreated hypertensive patients separated according to either *CYP11B1* or *CYP11B2* genotypes or the main *CYP11B* haplotypes. The sensitivity of their aldosterone response to an extremely low dose of exogenous ACTH, following suppression of endogenous levels with the synthetic

steroid dexamethasone, would determine if there is a link between ACTH drive and aldosterone synthesis. However, the impact of a small decrease in 11β-hydroxylase efficiency could be more fully determined in an animal model. For example rats given a very low dose of the 11β-hydroxylase inhibitor, metyrapone, would enable determination of the full effects of this enzyme inefficiency on adrenal structure and function, corticosteroid levels and blood pressure.

Therefore although an explanation for the paradoxical link between *CYP11B2* variants and 11β-hydroxylase function has been provided, the causative link with hypertension and a raised ARR remains to be determined.

APPENDICES

Appendix 1: Nucleotide sequences of human *CYP11B1* and *CYP11B2*

Nucleotide Sequence

The nucleotide sequences for human *CYP11B1* and *CYP11B2* were obtained from the Entrez Nucleotide database. The accession numbers for these sequences are as follows:

	CYP11B1	*CYP11B2*
5' Flanking Region and exon 1	D10169	D10170
Exon 1 and 2	M32863	D13752
Intron 2	X85218	D13752
Exons 3 to 8	M32878	D13752
Exon 9 and 3'UTR	M32879	M32881

Exons are shown in bold and the untranslated regions and intron in lower case. The oligonucleotides used for PCR and sequencing, listed in **appendix 2**, are indicated by their name above the relevant highlighted sequence. Where oligonucleotide sequences overlap the second sequence is underlined as is the primer name. Sense and antisense oligonucleotides are indicated by > and < respectively.

CYP11B1

```
     B15'UTR >            (B15'7-32 >)
   1 tccttcgcat cccttgtaag ttggattcct aagtatttta ttctctttga

  51 agcaattgtg aatgggagtt cactcatgat ttggcctctc tgtttgtctg

 101 ttaagggtgt ataagaatgc ttgtgatttt tgtacattga ttttgtatcc

 151 tagagcttgc tgaagttgct tatcagctta aggagatttt gggctgagac
                                                < B15'258-238
 201 aatggggttt tctagatata caatcatgtc cgtctgccaa acagagacaa

 251 tttgacttcc tcttttccta attgaatacc ctttatttcc ttctcctgcc

 301 taattgccct ggccagaact tccaacacta tgttgaatag gagtggtgag
                                  < B15'393-369          F81092 >
 351 agagggcatt cctgtcttgt accagctttc acagggaatg cttccagttt
```

401 ttgaccattc agtatgatat tggctgtggg tttgccatag atagctctta

451 ttattttgag atacgtccca tcaataccta atttattgag agtttttagc

501 gtgaaggttg ttgaattttg tcaaaggcct tttctgcatc tattgagata

551 atcatgtggt ttttgtcttt ggttctgttt atatgctgga ttacatttat

F81093 >

601 tgatttgcat atattgaacc agccttgcat cccagggatg aagcccactt

651 gatcatggtg gataagcttt ttgatgtgct gctggattcg gtttgccagt

701 attttattga ggatttttgc atcaatgttc atcaaggata ttggtctaaa

751 attctctttt ttggttgtgt ctctgcccgg ctttggtatc aggatgatgc

801 tggcctcata aaatgagtta gggaggattc cctctctttc tattgattgg

F81094 >

851 aatagtttca gaaggaatgg taccagttcc tccttgtacg tctggtataa

901 ttcggctgtg aatccatctg gtcatggact ctttttggtt ggtaatctat

951 tgattattgc cacaatttca gatcctgtta ttggtctatt cagagattca

1001 acttcttact ggtttagtct tgggagagtg tatgtgtcga ggaatttatc

1051 catttcttct agattttcta gtttatttgc gtagaggtgt ttgtagtatt

F81095 >

1101 ctctgatggt agtttgtatt tctgtgggat cggtggtgat atccccttta

1151 tcatttttta ttgcatctat ttgattcttc tctcttttt tctttattag

1201 tcttgctagc ggtctatcaa ttttgttgat cctttcaaaa aaccagctcc

1251 tggattcatt aatttttga agggtttttt gtgtctctat ttccttcagt

1301 tctgcactga ttttagttat ttcttgcctt ctgctagttt tgaatgtgtt

1351 tgctcttgct tttctagttc ttttaattgt gatgttaggg tgtcagtttt

1401 ggatctttcc tgctttctct tgtgggcatt tagtgctata aatttccctc

1451 tacacactgc tttgaatgtg ttccagagat tctggtatgc tgtgtctttg

1501 ttctcgttgg tttcaagaac atctttattt ctgccttcat tttgttacgt

1551 acccagtagt cattcaggag caggttgctc agtttccatg taattgagcg

F81097 >

1601 gttttgagtg agtttcttaa tcctgagttc tagtttgatt gcactaaaat

1651 ttttaaaaag taaaaaaaat acatgtggtt taatacaatt catgccaact

< B1prom-260(as)

1701 cattccctcg ttttttgcta taaaccttgc aaggagatga ataatccaag

1751 gctcttggat aagataaggg ccccatccat cttgctcctc tcagccctgg

1801 aggaggaggg agagtccttt tcccctgtct acgctcatgc acccccaatg

F31463 >

1851 agtccctgcc tccagccctg acctctgccc tcggtctctc aggcagatcc
1901 agggccagtt ctcccatgac gtgatccctc ccgaaggcaa ggcaccaggc

1951 aagataaaag gattgcagct gaacagggtg gagggagcat tggaATGGCA Ex1
< B9865
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2051 AAGGGCACAG GCACTGGGCA CGAGAGCCGC CCGGGTCCCC AGGACAGTGC

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2601 cttggcccca cagGTACGAT TTGGGAGGAG CAGGCATGGT GTGTGTGATG Ex2

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3351 ccgttggtca ggtgggcagg gagaggctga tgcttggagt cagtcacctg

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B4250 >

4851 gaggcccggt tcccttgtgc tcagcagtgc atcctccccg cag**CCAGCAA** **Ex4**

4901 **CTTGGCTCTT TTTGGAGAGC GGCTGGGCCT GGTTGGCCAC AGCCCCAGTT**

4951 **CTGCCAGCCT GAACTTCCTC CATGCCCTGG AGGTCATGTT CAAATCCACC**

5001 **GTCCAGCTCA TGTTCATGCC CAGGAGCCTG TCTCGCTGGA CCAGCCCCAA**

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5451 **GGCGGAGCTC CTGTTGAATG CGGAACTGTC GCCAGATGCC ATCAAGGCCA**

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5901 ag**ACGGTGTT TCCCTTGCTG ATGACGCTCT TTGAGCTGGC TCGGAACCCC** **Ex6**

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6051 **CCCTCAAGGA GACCTTGCG**g tgggtgctgg ctgaggcctc cctgtggccc

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6651 CCGCCTTGTT CCCGAGGCCT GAGCGCTATA ACCCCCAGCG CTGGCTAGAC

6701 ATCAGGGGCT CCGGCAGGAA CTTCTACCAC GTGCCCTTTG GCTTTGGCAT

6751 GCGCCAGTGC CTTGGGCGGC GCCTGGCAGA GGCAGAGATG CTGCTGCTGC

6801 TGCACCATgt gagcaggccc gggctgggga ggggcctggg cggggtctgg

6851 gcagcatggg cggggcttga gcaatgtggg actggcctcg gcagagtggg

6901 agtggcctgc atgtttcctg gactgggcag agcgggtact gggagaacct

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7051 ggggaagtcg ggtggagcct gtacaggata gtggggcttg ggcaatacct

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7201 aactttgagg gtctgagaag gctgcaccac gtgcatgggc tgcggaccaa

7251 gccagatgga aacccggctt ctgtcctagG TGCTGAAACA CCTCCAGGTG Ex9

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7451 caggccaccc ctcttctctc ccacatgcac agcttcctga gtcacccctc

7501 tgtctaacca gccccagcac aaatggaact cccgagggcc tctaggacca

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351 cctgaggtca ggagttcgag actagcctgg ccaacacggt gaaaccccgt

401 ctctactaaa aatacaaaaa ttagctgggc gtggtggtgg cacatgcctg

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501 agacagaggt cgcagtgagc cgagatcaca ccattgcact ctagcctggg

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601 ataataaaaa taaataaata aaagccagaa agtgtatttg atgatcatag

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1951 tgatatgttt ccagagcagg ttcctgggtg agataaaagg atttgggctg

2001 aacagggtgg agggagcatt gga**ATGGCAC TCAGGGCAAA GGCAGAGGTG** **Ex1**

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2101 TAGAGCCGCT CGGGCCCCTA GGACGGTGCT GCCGTTTGAA GCCATGCCCC

2151 AGCATCCAGG CAACAGGTGG CTGAGGCTGC TGCAGATCTG GAGGGAGCAG

2201 GGTTATGAGC ACCTGCACCT GGAGATGCAC CAGACCTTCC AGGAGCTGGG

2251 GCCCATTTTC AGgtaaagcc ctccctggcc ctcgctggga acacccagat

2301 ccctgcccct gctgcccagg accctgccag gcactcagca ctgccattcc

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2551 gcagggaggg tggacaggag acactttgga ttgggactgc agggtggggc

2601 cacgagggac atgaccccgt ccagcagggc ctcctgcttg gccccacagG

2651 **TACAACTTGG GAGGACCACG CATGGTGTGT GTGATGCTGC CGGAGGATGT** **Ex2**

2701 GGAGAAGCTG CAACAGGTGG ACAGCCTGCA TCCCTGCAGG ATGATCCTGG

2751 AGCCCTGGGT GGCCTACAGA CAACATCGTG GGCACAAATG TGGCGTGTTC

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4601 ctggcctctg tag**GAATGGG CCTGAATGGC GCTTCAACCG ATTGCGGCTG** **Ex3**

4651 AACCCAGATG TGCTGTCGCC CAAGGCCGTG CAGAGGTTCC TCCCGATGGT

4701 GGATGCAGTG GCCAGGGACT TCTCCCAGGC CCTGAAGAAG AAGGTGCTGC

4751 AGAACGCCCG GGGGAGCCTG ACCCTGGACG TCCAGCCCAG CATCTTCCAC

4801 TACACCATAG AAGgtgtggg ccatgcggga aggtccagcc ccagagaccc

4851 tggagtggcc agggatgggg atggaggact gaagggagtg tggggaggca

4901 gccaggaggc ctggggctgc cttgtgctca gcagtgcatc ctccccgcag

4951 **CCAGCAACTT AGCTCTTTTT GGAGAGCGGC TGGGCCTGGT TGGCCACAGC** **Ex4**

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6651 aatctggggc tgataaacag cgtcacccag cagcccattc ccctgcacct

6701 gctcttcctc cccctcaagg acagggagct cttcttcctc tggaatccct

6751 cttcaacgcc ctggggatta acgtggggca tgtccttctg cgctcggggc

6801 tgcttaagtt aggggaggtt tggctgggct cagcaggtgc aaggaagcac

6851 ttcctacgac ctgggcttcc catggatctg ggacctctgc ggggtcttcg

6901 gtaggaaggg tgctgagagc acagggagcc ccatccagct gaggaccctt

6951 tctgtggatg cccccacctc cagGCTCTAC CCTGTGGGTC TGTTTTTGGA Ex7

7001 GCGAGTGGTG AGCTCAGACT TGGTGCTTCA GAACTACCAC ATCCCAGCTG

7051 GGgtgagtga gccccacacc cctcgagctg agaacctccc tccccagtca

7101 ttccctgatc cctgctctgc accgtccgca gACATTGGTA CAGGTTTTCC Ex8

7151 TCTACTCGCT GGGTCGCAAT GCCGCCTTGT TCCCGAGGCC TGAGCGGTAT

7201 AATCCCCAGC GCTGGCTAGA CATCAGGGGC TCCGGCAGGA ACTTCCACCA

7251 CGTGCCCTTT GGCTTTGGCA TGCGCCAGTG CCTCGGGCGG CGCCTGGCAG

7301 AGGCAGAGAT GCTGCTGCTG CTGCACCACG TAAGCAGGCC TGGGGGCGGG

7351 GGCGGGACCT GGGCAGCAGA GGCGGGACCT GCACACTGGG GGCGGGGCTT

7401 GCATGGTGTg attgacacct gggaacagtg gatggggcct tggttggttg

7451 aggtcggcgt gaccagggag gatctgtgct gagcaagaca gggtaggatc

7501 tgggtgaggt tgcttctaaa cattgaaatg gggactaggg gagtggggtg

7551 gagcctgtac agaataatgg ggcttgggca agacctgggc aggattcagt

7601 ctgggcctgg tccgcaaggt ggggctggtc agaaatggga taggttgggg

7651 cccaggctgc tgctcccccc tcagcataat tgttgcacct gggacgatgg

7701 gaggaagctg ccccaggtcc atgggctact gaccaggcca gatggaaacc

7751 cagcctctgt cctagGTGCT GAAGCACTTC CTGGTGGAGA CACTAACTCA Ex9

B2 3'UTR >

7801 AGAGGACATA AAGATGGTCT ACAGCTTCAT ATTGAGGCCT GGCACGTCCC

7851 CCCTCCTCAC TTTCAGAGCG ATTAACTAGt cttgcatctg cacccagggt

```
7901 cccagcctgg ccaccagctt ccctctgcct gaccccaggc cacctgtctt
          < M6641
7951 ctctcccacg tgcacagctt cctgagtcac ccctctgtcc agccagctcc

8001 tgcacaaatg gaactcccca gggcctccag gactggggct tgccaggctt

8051 gtcaaatagc aaggccaggg cacagctgga gacgatcttg ctggcagggc

8101 ctggccttgt ccccagcccc acctggcccc ttctccagca agcagtgccc

8151 tctggacagc ttgactctac tcctcccagc gctggctcca ggctcctcat

8201 gaggccatgc aagggtgctg tgattttgtc ccttgccttc ctgcctagtc

8251 tcacatgtcc ctgtccctct cgccctggcc agggcctctg tgcagacagt

8301 gtcagagtca ttaagcggga tcccagcatc tcagagtcca gtcaagttcc

8351 ctcctgcagc ctgaccccag gcagctcgag catgccctga gctctctgaa

8401 agttgtcacc cagaaatacg atcctgcagg gtagactaaa aaggcccctg

8451 tggtcactta tactgacaca ttttaagtga tacaactgag tctcgagggg

8501 cgtgtgttcc ccagctgatc atgtcagcct catgccccag gcctcgtctt

8551 tcatggacca ggtcttgttc aagcagcgag tgttgggtcc tctgcttcct

8601 gagctgtccc ctggaaaagg tcccgaggat gctgtcagga gatggaagag

8651 tcatgtgggg tgggaacctg gggtgtggtt ccagaaatgt ttttggcaac

8701 aggagagaca ggattgggcc aacaaggact cagatgagtt tattgactca
                                                  < B2 3'UTR
8751 ttcctctgga agatacgcag c.....................ctcccct
9251 agtcagcaca tc
```

Appendix 2: Sequences of oligonucleotide primers

In each case (s) indicated the sense primer and (as) indicated the antisense primer

Exon	SSCP Primer Sequence	Amp Size (bp)	Rest. Enzyme	Fragment Sizes (bp)
1	S 5′-AATGAGTCCCTGCCTCCAGC-3′ AS 5′-GAATGGCAGTGCTGAGTGCC-3′	473	Xho11	49,260,164
2	S 5′-TTTGGATTGGGACTGCAGGG-3′ AS 5′-CCCACCCTGCTCCCAGCTTC-3′	260	Hha1	77,183
3	S 5′-TGGCCACTCCAGGGTCTCTG-3′ AS 5′-CTGCAGGCCGATTCCCCTTG-3′	349	Hha1	115,234
4	S 5′-CCTTGTGCTCAGCAGTGCAT-3′ AS 5′-GTGGTGGAGAGGGAGAAATT-3′	306	Stu1	210,96
5	S 5′-AGGAGGACACTGAAGGATGTT-3′ AS 5′-GACACGTGGGCGCCGTGTGAC-3′	312	Rsa1	183,189
6	S 5′-TCTGACCCTGCAGCTGTGTCT-3′ AS 5′-AGGGCCACAGGGAGGCCTCA-3′	224	N/A	224
7	S 5′-AATGACTGGGGAGGGAGGTT-3′ AS 5′-TGGATGCCCCCACCTCCAGG-3′	147	N/A	147
8	S 5′-CCCTCGAGCTGAGAACCTCC-3′ AS 5′-CCAGTCCCACATTGCTCAAG-3′	262	Hae111	119,143
9	S 5′-CTGTTCCCCCTTCAGCATAAT-3′ AS 5′-GAGACGTGATTAGTTGATGGC-3′	224	N/A	224

Table 1. SSCP sense (S) and antisense (AS) primer sequences, amplicon sizes (Amp size) and restriction digests

CYP11B1 MUTATIONS			
Exon	**Amino acid Change**	**Sequence**	
1	R43Q	Sense	5`-GAA GCC ATG CCC CAG CGT CCA GGC AAC-3`
		Antisense	5`-GTT GCC TGG ACG CTG GGG CAT GGC TTC-3`
2	L83S	Sense	5`-CAG GTA CGA TTC GGG AGG AGC AGG CAT G-3`
		Antisense	5`-CAT GCC TGC TCC TCC CGA ATC GTA CCT G-3`
2	H125R	Sense	5`-GAC ACC ATC GTG GGC GCA AAT GTG GCG TGC-3`
		Antisense	5`-GCA CGC CAC ATT TGC GCC CAC GAT GTT GTC-3`
3	P135S	Sense	5`-CTT GTT GAA TGG GTC TGA ATG GCG CTT C-3`
		Antisense	5`-GAA GCG CCA TTC AGA CCC ATT CAA CAA G-3`
3	F139L	Sense	5`-GGC CTG AAT GGC GCC TCA ACC GAT TGC G-3`
		Antisense	5`-CGC AAT CGG TTG AGG CGC CAT TCA GGC C-3`
3	L158P	Sense	5`-GTG CAG AGG TTC CCC CCG ATG GTG GAT G-3`
		Antisense	5`-CAT CCA CCA TCG GGG GGA ACC TCT GCA C-3`
3	L186V	Sense	5`-GGG GAG CCT GAC CGT GGA CGT CCA GCC-3`
		Antisense	5`-GGC TGG ACG TCC ACG GTC AGG CTC CCC-3`
3	T196A	Sense	5`-CAG CAT CTT CCA CTA CGC CAT AGA AGC CAG C-3`
		Antisense	5`-GCT GGC TTC TAT GGC GTA GTG GAA GAT GCT G-3`

Table 2. Primers for site-directed mutagenesis

SEQUENCING PRIMERS		
Name	**Exon**	**Sequence**
V6776	$pCMV_4$ (s)*	5`TAG TGA ACC GTC AGA ATT G 3`
V6777	$pCMV_4$ (as)*	5`TAG AGG ACA CTA GTC AGA C 3`
N4019	Exon 2 (s)	5`AGA CAA CAT CGT GGG CAC AAA TG 3`

* primers flanking either side of the $pCMV_4$ cloning site

Table 3. Sequencing primers for $pCMV_4$-B1 wildtype and mutant constructs

	Oligonucleotide Seq (5′-3′)
HB1 sense	TGC GCG TGT TCC TCT ACT CT
HB1 antisense	GTG CAG AGA CGT GAT TAG TTG ATG
HUMB1FL	TAC AGC TTC ATA TTG AGG CCC AGC A-FL
HUMB1LC	LC-TTC CCC CTC CTC ACC TTC AGA GC

FL, Fluorescein; LC, LightCycler Red705 dye.

Table 4: Lightcycler primers and probes for QRT-PCR

Primer	Sequence	PCR
B15'UTR (s)	5`-TCC TTC GCA TCC CTT GTA GTA AGT T- 3`	B1 5'
B1Exon9 (as)	5`-TAG TTG ATG GCT CTG AAG GTG A- 3`	to Ex9
M6635 (s)	5`-CTG TTC CCC CTT CAG CAT AAT- 3`	B1
B13'UTR+500 (as)	5`-CTA TTC CAG GGC GAC AAC TTT- 3`	3'UTR
T8303D (s)	5`-GAA TTC TGC ATC CTG TGA AAT TAT C- 3`	B2 5'
F33915 (as)	5`-GTG GTG GAG AGG GAG AAA TT- 3`	to Int4
T2138 (s)	5`-ATT TGG GTG TCG GGG CAG TCT-3`	B2 Ex5
M6641 (as)	5`-CTG TGC ACG TGG GAG AGA AGA-3`	to Ex9
B2 3'UTR (s)	5`-ATA TTG AGG CCT GGC ACG TC- 3`	B2
B2 3'UTR (as)	5`-GAT GTG CTG ACT AGG GGA G- 3`	3'UTR

Table 5. Primer pairs for PCR amplification of the entire *CYP11B1* and *CYP11B2* genes

CYP11B1 SEQUENCING PRIMERS		
Primer	**Exon**	**Sequence 5′ – 3′**
B1Prom-1750(as)	5'	AAG TCA AAT TGT CTC TGT TTG
F81092 (s)	5'	CAC AGG GAA TGC TTC CAG TTT TTG
F81093 (s)	5'	CCA AGG GAT GAA GCC CAC TTG AT
F81094 (s)	5'	AAT GGT ACC AGT TCC TCC TTG TAC
F81095 (s)	5'	CTC TGA TGG TAG TTT GTA TTT CTG T
F81097 (s)	5'	TAA TCC TGA GTT CTA GTT TGA TTG C
B9865 (as)	5'	CTG CCT TTG CCC TGA GTG CC
F31463 (s)	1	AAT GAG TCC CTG CCT CCA GC
Y6266 (as)	1	CCC ACC CTG CTC CCA GCT CT
Y6265 (s)	2	TTT GGA TTG GGA CTG CAG GG
B1Int2 (3280) (s)	Int2	AGA AGC AAG GCA CGA GGT GG
B1Int2 (3580) (s)	Int2	GGA CCC AGG AAG GCA AGT CCA
B1Int2 (3690) (s)	Int2	GGC TGC CAT CAC TGT CAA CA
N4020 (as)	Int2	TTC AGC CGC AAT CGG TTG AAG
Y6263 (s)	3	CTG CAG GCC GAT TCC CCT TG
B4251 (as)	3	GTG GTG GAG AGG GAG AAA TT
B4250 (s)	4	CCT TGT GCT CAG CAG TGC AT
M6643 (as)	4	GAC ACG TGG GCG CCG TGT GAC
M6642 (s)	5	AGG AGG ACA CTG AAG GAT GTT
F42299 (as)	5	AGG GCC ACA GGG AGG CCT CA
F42117 (s)	6	TCT GAC CCT GCA GCT GTG TCT
Y6260 (as)	6	AAT GAC TGG GGA GGG AGG TT
Y6259 (s)	7/8	TGG ATG CCC CCA CCT CCA GG
M6635 (s)	9	CTG TTC CCC CTT CAG CAT AAT
B1Ex9as (as)	9	TAG TTG ATG GCT CTG AAG
B13'UTR+500 (as)	3'	CTA TTC CAG GGC GAC AAC TTT

Table 6. Primers for the Sequencing of the entire *CYP11B1* gene

	Primer	Sequence
PCR 1	Sense (B15'UTR)	5′-TCC TTC GCA TCC CTT GTA AGT T-3'
	Antisense (B1prom-260)	5′-CTT GGA TTA TTC ATC TCC TTG CAA GG-3'
PCR 2	Sense (B15'7-32)	5′-GCA TCC CTT GTA AGT TGG ATT CCT AA-3'
	Antisense (B15'393-369)	5′-AAG CAT TCC CTT TGA AAA CTG GTA C-3'

Table 7. Nested PCR primers for amplifying the region surrounding the -1889 G/T and -1859 A/G polymorphisms

The polymorphisms were then genotyped by sequencing using the primer B15'258-238 (see **table 5**).

Base Change SNP		SequencePrimeSequence
-1889	(S)	5′-CTC TGT TTG TCT GTT AAG TGT GTA TAA GAA TGC TTG TG-3'
G to T	(AS)	5′-CAC AAG CAT TCT TAT ACA CAC TTA ACA GAC AAA CAG AG-3'
-1859	(S)	5′-GCT TGT GAT TTT TGT ACG TTG ATT TTG TAT CCT GAG AC-3'
A to G	(AS)	5′-GTC TCA GGA TAC AAA ATC AAC GTA CAA AAA TCA CAA GC-3'

Table 8. Site-directed mutagenesis primers for *CYP11B1* 5'UTR polymorphisms

Appendix 3: Reagents

Unless otherwise stated all standard chemicals and reagents were purchased from Sigma (Sigma-Aldrich Chemical Co. Ltd, Poole, Dorset, UK).

Bacterial media and plates

LB medium	10g tryptone, 5g yeast extract, 10g NaCl to a final litre with deionised water, adjust to pH 7.0 with NaOH. Autoclave
LB agar	10g NaCl, 10g Tryptone, 5g yeast extract, 20g of agar, adjust to pH 7.0 with NaOH. Autoclave
LB-amp plates	1 litre of LB agar, add 400 µl of filter-sterilized ampicillin

Buffers

TE buffer	10mM Tris-HCl, 1mM EDTA, pH 7.5
TAE buffer	40mM Tris-acetate, 1mM EDTA, pH 8.0
Loading dye	1.5g Ficoll, 25mg Bromophenol Blue, 25mg Xylene Cyanol

Site directed mutagenesis

Quick change site-directed mutagenesis kit (Stratagene)

- Pfu DNA polymerase (2.5U/µl)
- 10X reaction buffer
- Dpn I restriction enzyme (10U/µl)

Reagents not supplied in the kit:

- Primers (see **table 2 and 8, appendix 2**)
- NZY+broth — 16g of NZM broth, 5g of yeast extract, 12.5ml of 1M MgCl2, 12.5ml of 1M MgSO4, 10ml of 2M filter-sterilized glucose solution per litre.
- LB-amp agar plates — (see above).

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